INTERCONTINENTAL DISJUNCTIONS IN BRYOPHYTES: SYSTEMATIC AND BIOGEOGRAPHIC STUDIES IN ORTHOTRICHEAE (ORTHOTRICHACEAE, BRYOPSIDA)

Disyunciones intercontinentales en briófitos: estudios sistemáticos y biogeográficos en Orthotricheae (Orthotrichaceae, Bryopsida)

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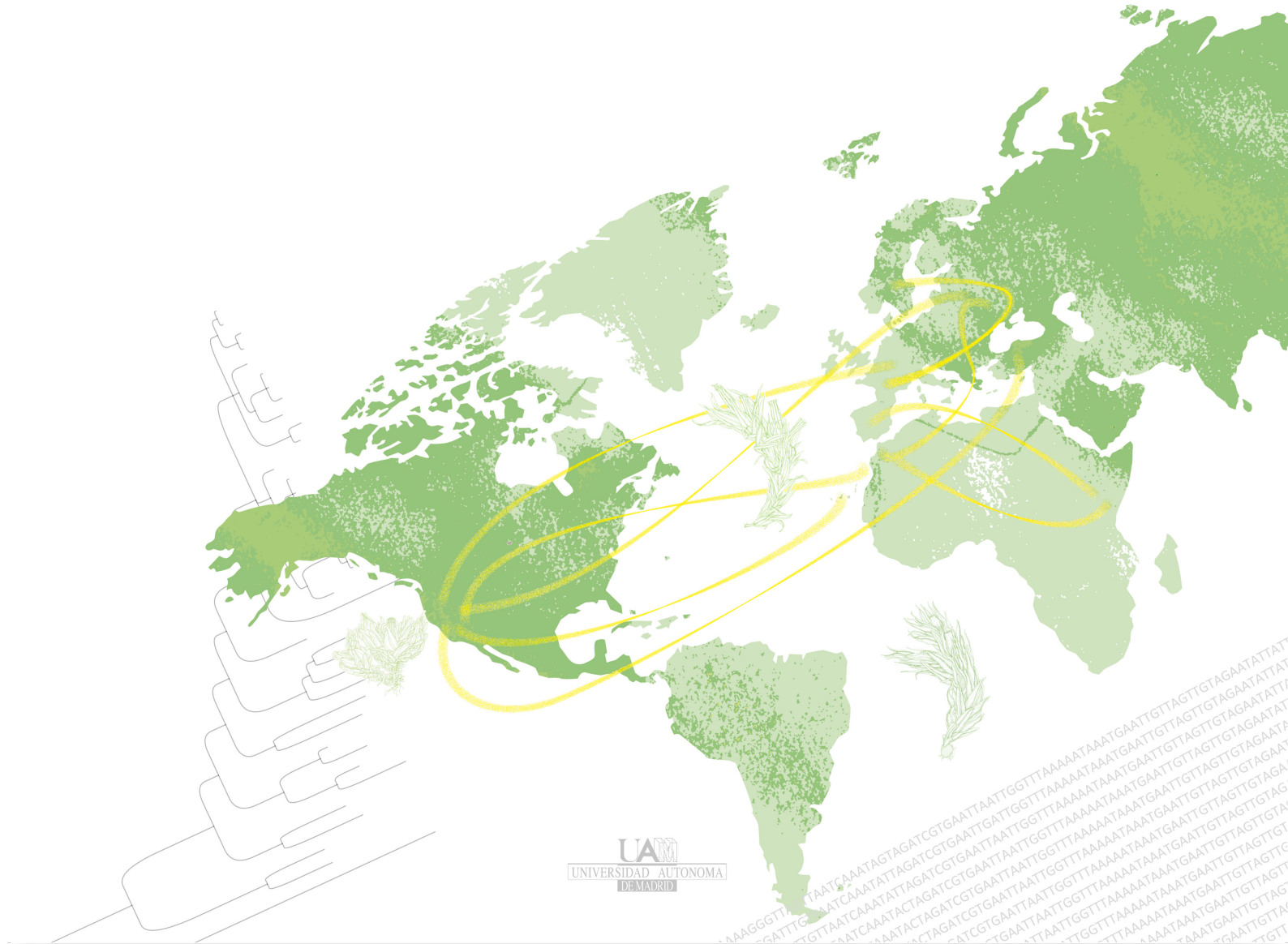
PhD Thesis

2017



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FACULTAD DE CIENCIAS

Departamento de BIOLOGÍA

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Thesis submitted to the
Universidad Autónoma de Madrid for the degree of
International Doctor of Philosophy for

BEATRIZ VIGALONDO GARCÍA
2017

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Memoria presentada para optar al grado de Doctora en Ciencias dentro del Programa de Doctorado 1393/2007 "Biología y Ciencias de la Alimentación"
por

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Madrid, Junio de 2017

A mi familia

A Juancho

Beatriz Vigalondo

Intercontinental disjunctions in bryophytes: systematic and biogeographic studies in Orthotricheae (Orthotrichaceae, Bryopsida)

Cover design and layout by Beatriz Vigalondo

Drawings of the cover and covers of chapter 3.1, 3.2 and 3.4 by Inma Guijarro, from Lara, F., & Garilleti, R. (2014) *Orthotrichum*. In: Guerra J, Cano MJ, Brugués M, eds. *Flora Briofitica Ibérica. Volumen V. Orthotrichales*. Murcia: Universidad de Murcia - Sociedad Española de Briología, 50–135.

Drawing of the cover and cover of chapter 3.3. from Lewinsky-Haapasaari, J. & Norris, D.H. (1998) *Orthotrichum shevockii* (Orthotrichaceae), a new moss species from the Southern Sierra, California. *The Bryologist*, 101, 435–438.

Chapters 3.1 and 3.2 may differ slightly from the original publications.

This research has been possible due to the "Formación de Personal Investigador" fellowship (FPI program 2012) of the "Ministerio de Economía y Competitividad" (reference BES-2012-051976) and the research project CGL2011-28857 of the "Ministerio de Ciencia e Innovación" from the Spanish Government.

The same FPI program financed two research stays at international institutions:

- Institute of Botany, Department of Biology, Ecology and Evolution, University of Liège, Liège, Belgium. September – December 2014 (ref: EEBB-I-14-08081). Supervisor: PhD Alain Vanderpoorten.

- College of Liberal Arts and Sciences, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs (Connecticut), USA. May – August 2015 (ref: EEBB-I-15-09739). Supervisor: PhD Bernard Goffinet.

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Abstract

Bryophyte species tend to display wide distribution ranges that often span over more than one continent. Furthermore, in comparison with angiosperms, bryophytes show a considerable lower rate of endemism. This raises interesting questions regarding the origin of their distributions and the evolutionary processes that rule these plants. Bryophytes also represent a taxonomically challenging group due to their less complex morphologies. Currently, the taxonomic and biogeographic hypotheses formulated on the basis of morphological approaches are being revised in the light of the data obtained from molecular analyses, and especially from integrative taxonomic approaches. Despite the numerous studies so far realized, it is not yet possible to generalize on the causes that have originated the present distribution ranges of bryophytes. Long distance dispersal is increasingly supported as the main factor shaping current bryophytes distributions, but fragmentation and continental drift have also been documented in several occasions. High dispersal capacities of bryophytes have been proposed as one of the factors leading to long distance dispersal, and to the low endemism rates of bryophytes compared to angiosperms. However, several studies suggest that the underestimation of bryophytes diversity, due to taxonomical shortcomings or the existence of cryptic species, may also be the reason underlying some current broad distribution ranges.

The tribe Orthotricheae, and in particular the genera *Orthotrichum* and *Lewinskya*, are among the most diverse and complex groups of mosses from a taxonomic, phylogenetic and biogeographic point of view. However, no complete molecular phylogeny of the group has been yet performed, and only one species, *O. handiense*, has been included in biogeographical or phylogeographic studies. This doctoral thesis aims to provide new on the biogeographic patterns of the genera *Orthotrichum* and *Lewinskya*, focusing on three main species, *Lewinskya acuminata*, *L. affinis* and *Orthotrichum shevockii*, which may serve as a basis for a better understanding of the evolutionary and biogeographic processes of the tribe Orthotricheae, but also of extant bryophytes in general. In this sense, this research also intends to add new evidence of the involvement of the different mechanisms that shape bryophytes distributions, namely the long-distance dispersal *versus* the remote fragmentation of continuous areas, or even if new cases of parallel or convergent evolution

can be inferred for this group of organisms. In addition, this work undertakes the analysis of large disjunct distributions, to assess if the populations at both extremes of the disjunction represent the same taxa, or conversely, if they are distinct species; and in this last case, if they are true cryptic species, or species for which morphological characters that could allow for their clear discrimination have been overlooked. Finally, through the assessment of the relationships existing among the groups considered in the study, this thesis aims to contribute to clarify the phylogeny of the two involved genera: *Orthotrichum* and *Lewinskya*.

The overall methodology of this thesis follows an integrative taxonomic approach, combining different molecular and morphological analyses, and considering additional available geographic information of the different taxa included. In the studies of *Lewinskya acuminata*, *L. affinis* and *Orthotrichum shevockii*, phylogenetic inferences are contrasted with different multivariate statistical analyses of morphological traits, including molecular species delimitation analyses for the case of *L. affinis*. Next-Generation Sequencing tools are implemented to obtain the mitochondrial genome of two species of *Orthotrichum*, *O. diaphanum* and *O. macrocephalum*, with the final purpose of finding new variable molecular markers for phylogenetic and phylogeographic analyses of species belonging to the tribe Orthotricheae, and testing the phylogenetic relationships of *Orthotrichum* and *Lewinskya*.

The results obtained in this study support the existence of a considerable gap of knowledge respect to bryophytes diversity and distributions, as well as the impossibility of assuming the presence of general patterns on bryohpytes distributions. From one side, this study describes two new independent and uncommon cases of intercontinental disjunctions within the tribe Orthotricheae. The first one involves *Lewinskya acuminata*, so far known as a Mediterranean-Macaronesian species, whose presence is reported for California and Ethiopia. The second case confirms the presence of the Californian species *Orthotrichum shevockii* in Macaronesia, particularly in Tenerife Island. The results suggest that the disjunctions of both species have their origins in long distance dispersal processes, adding evidence to the important role of this type of events in modeling the distribution patterns of extant bryophytes.

Conversely, the up-to-date wide disjunct distribution of the species *Lewinskya affinis* is discarded. Species delimitation analyses reveal that *L. affinis* is actually a complex of species, including two reinstated synonyms and four new species, each of them showing

narrow and restricted distributions, overlapping in most cases, and none of them disjunct. Integrative taxonomic analyses support that the overlooked diversity within *L. affinis* is due to both taxonomical shortcomings and the existence of cryptic species. Detailed morphological re-evaluation of the seven identified species allows discriminating each of them by a specific combination of traits, although distinctions are not always simple. Moreover, the overall morphological similarity of the species of this complex is not attributable to convergent evolution processes as recently suggested for two other species complexes of *Orthotrichum*, since in this case the species integrate a natural monophyletic group, and thus can be considered sibling species.

As for the taxonomy of the Orthotricheae, the currently proposed division of *Orthotrichum s.l.* in the genera *Orthotrichum s.str.* and *Lewinskya*, and the close relation of the latter with genus *Ulota*, are supported by the phylogenetic analyses performed with the complete mitochondrial genome of several species of these genera. However, further studies are needed to obtain a more complete phylogeny of this tribe. Additionally, the analyses of the mitochondrial genome at inters- and intraspecific level of *O. diaphanum* and *O. macrocephalum* reveal a low overall genetic variation along this genome. These results agree with those obtained using different chloroplast and nuclear markers for the studies of *L. acuminata*, *L. affinis* and *O. shevockii*. All of them point to the need of evaluating new tools, like Next-Generation Sequencing techniques, for the performance of future phylogeographic and species delimitation studies in the tribe Orthotricheae.

Resumen

Las especies de briófitos tienden a mostrar amplios rangos de distribución que a menudo abarcan más de un continente. Además, en comparación con las angiospermas, presentan una tasa de endemicidad considerablemente menor. Esto plantea interesantes cuestiones sobre el origen de las distribuciones de estas plantas y los procesos evolutivos que las rigen. Los briófitos también representan un grupo taxonómicamente difícil debido a que tienen una morfología menos compleja que otras plantas. Actualmente, las hipótesis taxonómicas y biogeográficas formuladas basándose en enfoques morfológicos están siendo revisadas en función de los resultados obtenidos a partir de análisis moleculares y, especialmente, a partir de aquellos basados en la metodología de la taxonomía integrativa. A pesar de los numerosos estudios realizados hasta la fecha, todavía no es posible generalizar sobre los factores que han determinado los patrones de distribución actuales de los briófitos. Cada vez más estudios apoyan la dispersión a larga distancia como el factor clave para entender dichos patrones, aunque la fragmentación y la deriva continental también se han documentado como claves en la génesis de diferentes disyunciones. La gran capacidad de dispersión de los briófitos se asume como una premisa indispensable para sustentar la dispersión a larga distancia estando, a su vez, relacionada con el menor número de endemismos entre los briófitos en comparación con las angiospermas. Sin embargo, varios estudios sugieren que la subestimación de la diversidad de briófitos, debido a errores taxonómicos o a la existencia de especies crípticas, puede igualmente estar relacionada con los supuestamente amplios rangos de distribución descritos actualmente para diferentes especies.

La tribu Orthotricheae, y en particular los géneros *Orthotrichum* y *Lewinskya*, se encuentran entre los grupos de musgos más diversos y complejos desde un punto de vista taxonómico, filogenético y biogeográfico. Sin embargo, aun no se ha llevado a cabo ninguna filogenia molecular completa del grupo, y sólo una especie, *O. handiense*, se ha incluido en estudios biogeográficos o filogeográficos. Esta tesis doctoral tiene como objetivo aportar nuevos datos sobre los patrones biogeográficos de los géneros *Orthotrichum* y *Lewinskya*, centrándose principalmente en tres especies, *Lewinskya acuminata, L. affinis* y *Orthotrichum shevockii*, de modo que puedan servir como base para una mejor comprensión de los procesos evolutivos y biogeográficos de la tribu Orthotricheae, pero también de otros

grupos de briófitos existentes en la actualidad. En este sentido, este estudio pretende a su vez ofrecer nuevos datos sobre el papel que pueden desempeñar diferentes mecanismos en la configuración de las distribuciones geográficas de los briófitos, en concreto, la dispersión a larga distancia frente a la fragmentación de áreas continuas, o si pueden confirmarse nuevos casos de evolución paralela o convergente en este grupo de organismos. Por otra parte, este trabajo comprende el análisis de especies con amplias distribuciones disyuntas, para evaluar si las poblaciones existentes en ambos extremos de la disyunción corresponden a los mismos taxones, o por el contrario, se trata de especies distintas. En este último caso, cabe determinar si son especies crípticas o especies para las que hasta la fecha se han ignorado caracteres morfológicos diagnósticos que podrían permitir la diferenciación taxonómica de forma clara. Por último, esta tesis contribuye a esclarecer las relaciones filogenéticas de los dos géneros objeto de estudio, *Orthotrichum* y *Lewinskya.*

La metodología general de esta tesis se enmarca dentro de la taxonomía integrativa pues se combinan diferentes análisis moleculares y morfológicos junto con la información geográfica disponible para los diferentes taxones estudiados. En los casos de *L. acuminata, L. affinis* y *O. shevockii*, los resultados obtenidos mediante inferencias filogenéticas se contrastan con el análisis estadístico y cualitativo de caracteres morfológicos, incluyendo además análisis moleculares de delimitación de especies en el caso de *L. affinis*. Asimismo, se han empleado técnicas de *Next-Generation Sequencing* para secuenciar el genoma mitocondrial de dos especies de *Orthotrichum, O. diaphanum* y O. *macrocephalum*, con el propósito de encontrar nuevos marcadores moleculares variables válidos para realizar análisis filogenéticos y filogeográficos de diferentes especies de la tribu Orhotricheae y para evaluar las relaciones filogenéticas de *Orthotrichum* y *Lewinskya*.

Los resultados obtenidos confirman que la diversidad taxonómica y los patrones corológicos de los briófitos son dos áreas de conocimiento aun por desarrollar en profundidad. Así, este estudio describe dos nuevos casos independientes y poco frecuentes de disyunciones intercontinentales dentro de la tribu Orthotricheae. El primero hace referencia a *Lewinskya acuminata*, hasta ahora conocida como una especie mediterráneo-macaronésica y que sin embargo aparece también en California y Etiopía. El segundo caso confirma la presencia de la especie Californiana *Orthotrichum shevockii* en Macaronesia, en concreto en la isla de Tenerife. Los resultados obtenidos sugieren que las disyunciones de ambas especies se deben a procesos de dispersión a larga distancia, incidiendo en el

importante papel que tiene este tipo de procesos en la configuración de los patrones actuales de distribución de los briófitos.

Por otro lado, en este trabajo se descarta la distribución disyunta hasta ahora descrita para la especie *Lewinskya affinis*. Los análisis de delimitación de especies revelan que *L. affinis* es en realidad un complejo multiespecífico, y sustentan la reivindicación de dos especies previamente descritas y la descripción de cuatro nuevas especies. Además, las siete especies escondidas bajo el concepto de *L. affinis s.l.* tienen una distribución reducida, varias de ellas son simpátricas, pero ninguna es disyunta. Los análisis de taxonomía integrativa realizados apoyan que la diversidad de especies asociada a *L. affinis* ha pasado desapercibida debido tanto a posibles deficiencias en las revisiones taxonómicas realizadas hasta la fecha, como a su condición de especies crípticas. La reevaluación morfológica detallada de las siete especies identificadas permite discriminar cada una de ellas mediante una combinación específica de caracteres, aunque las diferenciaciones no son siempre sencillas. Por otra parte, la similitud morfológica general que presentan las especies de este complejo no está relacionada con procesos de evolución convergente, como se ha sugerido recientemente para otros dos complejos de especies del género *Orthotrichum*, ya que en el caso del grupo de *L. affinis* todas las especies integran un grupo monofilético natural, de modo que pueden considerarse especies hermanas.

En cuanto a la taxonomía de la tribu Orthotricheae, la propuesta actual de división del género *Orthotrichum s.l.* en los géneros *Orthotrichum s.str.* y *Lewinskya,* así como la estrecha relación de éste último con el género *Ulota*, se corrobora a partir de los análisis filogenéticos realizados con el genoma mitocondrial completo de varias especies de estos géneros, aunque se necesitan más estudios para obtener una filogenia más completa de esta tribu. Además, los análisis del genoma mitocondrial de *O. diaphanum* y *O. macrocephalum* a nivel inter e intraespecífico, revelan una escasa variación genética a lo largo de este genoma. Estos resultados coinciden con los obtenidos utilizando diferentes marcadores cloroplásticos y nucleares en los estudios de *L. acuminata*, *L. affinis* y *O. shevockii*. Todo ello sugiere la necesidad de evaluar el uso de nuevas técnicas como las de *Next-Generation Sequencing* para abordar futuros estudios filogeográficos y de delimitación de especies en la tribu Orthotricheae.

Chapter

1

Introduction, aims and outline

Introduction

The need for describing biodiversity

Currently known biodiversity is around 1.8 million species, while it is estimated that the planet may host between 2 and 10 million (Costello *et al.,* 2013; Caley *et al.,* 2014). In the current context of global change along with the excessive habitat destruction, we are facing a global biodiversity crisis. Some of the ecosystems most affected by habitat destruction are among the most diverse and the ones in which more species are likely to be discovered. Estimates indicate that biodiversity will continue to decline over the 21st century (Pereira *et al.*, 2010). This means that many species have disappeared or will do so without having been yet described (Staab *et al.,* 2015).

Within this context, taxonomy arises as a crucial tool for documenting and describing biodiversity. If we ignore the extant organisms of our planet and their natural history, we will fail to preserve its biodiversity. Naming species and establishing their boundaries are the first steps for knowing and understanding the natural world and it is the key for biologists and scientists to communicate with each other. In fact, describing and naming a species "is an anchor for biological information about a species, including its taxonomic affinities, morphology, distribution and possible ecological role" (Tautz *et al.,* 2003). In other words, species are the basic units for biodiversity, biogeography, ecology or evolutionary biology, as well as for conservation biology.

Puzzlingly, despite the current numbers of biodiversity loss, we are also at the apogee of the discovery of new taxa, with about 18,000 species described each year since the beginning of the 21st century (Wheeler & Pennak, 2012; Costello *et al.,* 2013). The development of molecular techniques has undoubtedly promoted the increase of the rate of discovery of new species (Staab *et al.,* 2015). The current system of naming species was initiated by Linnaeus (1753), but even earlier, the description of organisms, and then species, was mainly based on morphological features. However, morphological species circumscriptions can

underestimate the number of actual species, since speciation is not always accompanied by evident morphological changes (Bickford *et al.,* 2007). In that sense, molecular tools have helped to reveal that behind concepts of morphologically uniform species, may actually exist a complex phylogenetic structure of what are called cryptic and sibling species (sensu Bickford *et al.,* 2007). Subtle morphological differences between close species can be easily overlooked in organisms whose structural simplicity and reduced morphologies difficult the finding of taxonomically relevant morphological traits. This is the case of bryophytes (Vanderpoorten & Shaw, 2010), although morphological characters available for species delimitation are usually higher in bryophytes than in many other small organisms (Medina N.G. *et al.*, 2011).

Bryophyte diversity, including mosses, liverworts and hornworts, encompasses about 14,000-25,000 species (Medina N.G. *et al.*, 2011; Shaw *et al.,* 2011). However, several factors may mislead the global bryophyte species richness (Konrat *et al.,* 2010; Magill, 2010). One of these factors is the traditional morphological species concept used for bryophytes. In the 19th century, bryophyte species were usually described based on geographical concepts, assuming that populations geographically distant should represent different species. Lately in the 20th century, bryologists started to move towards a morphological species concept, considering morphological divergence among populations as indicative of the presence of different species (Shaw, 2009). This approach also reduced considerably the number of taxa by combining disjunct taxa into broadly morphologically defined species that span several continents, assuming morphological uniformity (Shaw, 2001; Heinrichs *et al.,* 2009a). The development and increased use of molecular tools during the past 20 years for systematic and biogeographic studies have revealed incongruences between the morphological and genetic signals within several bryophyte taxa, suggesting that the true diversity, distribution ranges and relationships between species may be obscured if only morphological data were available (Vanderpoorten & Shaw, 2010). DNA based phylogenetic reconstructions of different bryophytes groups have uncovered cryptic species or overlooked diversity (Heinrichs *et al.,* 2009b, 2011; Patiño *et al.,* 2017b). Similarly, they have endorsed synonimizations (Vanderpoorten & Shaw, 2010; Vanderpoorten *et al.*, 2015), and revealed phylogenetic structure related to geographic rather than morphological patterns (Vanderpoorten & Long, 2006; Hedenäs, 2008; Fuselier *et al.,* 2009; Bechteler *et al.*, 2017). Likewise, molecular techniques have also allowed to re-evaluate species relationships or

even their re-circumscription to different genera (Stech *et al.,* 2012; Dong *et al.,* 2012; Heinrichs *et al.,* 2015).

Once noticed the utility of molecular tools for discovering new species, not only in bryophytes but also in all groups of organisms, the ideas of DNA barcoding and DNA taxonomy became popular. However, several critical voices upraised against the single use of molecular data for the correct identification and classification of biodiversity (for review see Goldstein & DeSalle, 2011). This debate gave light to what is known today as integrative taxonomy, i.e. to delimit, discover and identify species and other taxa using different available sources of information, including molecular and morphological data, but also biogeographic, ecological, physiological or behavioral ones (Will *et al.,* 2005; Dayrat, 2005). Using a purely genetic approach may misidentify or overlook species boundaries, especially in the case of founder events or recent speciation where processes of incomplete lineage sorting and hybridization may occur, but also in other circumstances (for review see Vanderpoorten & Shaw, 2010; Naciri & Linder, 2015). The same situation can also be reached if using only morphological data when dealing with cases of convergent morphological evolution (Edward and Knowles 2014), but especially in those groups of bryophytes and other morphologically simplified organisms with strong overlap in morphological traits among species or with high morphological plasticity (Stech *et al.,* 2013; Stenøien *et al.,* 2014). In such situations, phylogenies or other molecular data can give the clue to re-evaluate and identify taxonomical relevant characters (Medina R. *et al.,* 2012, 2013).

Integrative taxonomy in bryophytes studies is becoming popular, allowing the discovery of new species and complex of cryptic species (Renner *et al.,* 2013; Aranda *et al.,* 2014; Hedenäs *et al.,* 2014; Lang *et al.,* 2014; Draper *et al.,* 2015; Caparrós *et al.,* 2016, Sim-Sim *et al.,* 2017). However, still a considerable number of studies only use molecular data for species delimitation or discover new species that are not further described (Carter, 2012; Piñeiro *et al.,* 2012; Dong *et al.,* 2012; Stech *et al.,* 2013; Lang *et al.,* 2015; Rabeau *et al.,* 2017). Thus, there is still a substantial bias among the number of discovered species and those that are formally described with a proper re-evaluation of morphological traits (Pante *et al.,* 2014). As stated above, naming and describing the discovered species is the final purpose of taxonomy and of important need to achieve an accurate performance of biogeographic, ecological or biodiversity conservation studies.

Bryophytes biogeography

Following the morphological species concept in bryophytes, of general use along the 20th century, many species have been broadly defined, showing significant morphological variation and broad geographic distribution ranges that often span several continents (Schofield, 1988). Interestingly these ranges are frequently equivalent to those found within angiosperms at generic level (Shaw, 2001; Medina N.G. *et al.*, 2011).

Initially, plants disjunct distributions were explained by dispersal events. This idea was especially supported by Darwin's studies of the flora of oceanic islands compared to close continental landmasses (Winckworth, 2010). The acceptance of Wegener's plates tectonic theory promoted a quite radical shift towards continental drift and vicariance (Raven & Axelrod, 1974), relegating dispersal mostly for oceanic island species, since it was considered a very stochastic process and very difficult to observe directly. So, the continental fragmentation theory was long considered to be the main reason explaining the current distribution of many species. However, in the last decades, molecular data have brought back again the role of dispersal as a key factor modeling plants distribution (Queiroz, 2005; Cowie & Holland, 2006; Nathan, 2006; Sanmartín *et al.*, 2008). The possibility of dating molecular phylogenies reconstructions allowed biogeographers to contrast the previous hypothesis of vicariance. The calibration of molecular phylogenies with the fossil record, or with secondary sources such as the age of different geological events, revealed in several cases that the processes of continental fragmentation were older than the estimated age of diversification or divergence of species disjunct populations, being dispersal the most plausible option for interpreting those species distribution patterns (Queiroz, 2005; Crisp *et al.*, 2011; Christenhusz & Chase, 2013).

Bryophytes show a high potential for dispersal due to their high level of spores production and the small size of these spores (Frahm, 2008; Lönnell, 2011; Sundberg, 2013). Hence dispersal has always been considered as a factor contributing to the broad disjunct distributions of bryophytes (for review see van Zanten & Pócs, 1981), although during long time it was also relegated by vicariance (Schofield & Crum, 1972; Schuster, 1983). The use of molecular tools and divergence time estimates in bryophytes studies has sustained the role of vicariance and continental drift in some cases (Devos & Vanderpoorten, 2009 for review; Vanderpoorten *et al.*, 2010). However, an increasing number of studies, especially in the last

decade, support the importance of dispersal, and particularly long distance dispersal, for shaping different extant bryophyte species distributions and diversification (Piñeiro *et al.*, 2012; Lewis *et al.*, 2014b; Sun *et al.*, 2014; Shaw *et al.*, 2015; Kyrkjeeide *et al.*, 2016; Scheben *et al.*, 2016; Carter *et al.*, 2017).

Bryophytes diaspores are mainly dispersed by wind, although other vectors such as water (Hutsemékers *et al.*, 2013) and animal-mediated dispersal have also been reported (mammals, Barbé *et al.*, 2016; insects, Marino *et al.*, 2009). These latter would participate in local or regional dispersal (van Zanten & Pócs, 1981), although long distance dispersal favoured by birds has also been suggested (Lewis *et al.*, 2014a). Wind acts as the most common vector for long distance dispersal, since the smaller spores (<25 µm) are more likely to be dispersed by air currents through long distances (van Zanten & Pócs, 1981; Gillespie *et al.*, 2012; Wilkinson *et al.*, 2012). Different studies have shown that about 1% of the regional spore rain can have a trans- or intercontinental origin (Sundberg, 2013), and that prevailing winds might be modelling different disjunctions in bryophytes (Muñoz *et al.*, 2004). However, few species of bryophytes are really ubiquitous (Frahm, 2008). Bryophytes dispersal can be constrained by several factors like accessibility to transport vectors, the type of spore release mechanism (xero- or hygrocastique, Lazarenko, 1957) and other anatomical or physiological features of the sporangium (for review see Lönnell, 2011). Besides, prosperous colonization relies on many other factors like spores survival and resistance during and after transport, longevity or viability for their establishment in a suitable habitat (van Zanten & Pócs, 1981; Medina N.G. *et al.*, 2011). But even when all the conditions for successful dispersal and colonization "happen only once in a thousand or once in ten thousand years or more, this might be sufficient for an expansion of the range of the species involved" (van Zanten & Pócs, 1981).

One of the most striking biogeographic features of bryophytes is their extremely low rates of endemism and diversification when compared to angiosperms (Vanderpoorten *et al.*, 2010, 2011; Medina N.G. *et al.*, 2011). This situation can be produced by the dispersal capacities of bryophytes, since long distance dispersal can impede diversification by sustaining gene flow across wide geographic distances (Shaw *et al.*, 2014, 2015). However, these low rates of endemism and diversification might also be underestimated by the existence of cryptic species, or because the taxonomy of cryptogams in comparison with that of angiosperms is less complete. Thus, endemic taxa could probably remain to be described,

or to be reassessed for those species morphologically indistinguishable (Vanderpoorten *et al.,* 2011). Phylogenies of different bryophyte genera or complex of species have shown a distribution of lineages more related to geographic patterns than to morphological ones (Shaw *et al.,* 2005; Hutsemékers *et al.,* 2012; Dong *et al.,* 2012; Heinrichs *et al.,* 2013). They have also revealed numerous cases where one species thought to occur in disjunct regions, actually corresponded to several different species with narrower distribution ranges, due in some cases to cryptic speciation (for review see Shaw, 2001; Heinrichs *et al.,* 2009b), but in others to taxonomical shortcomings (Heinrichs *et al.,* 2010; Medina R. *et al.,* 2012, 2013). However, evidences for the opposite situation, suggesting broader species circumscriptions and synonimizations have also been found (for review see Vanderpoorten & Shaw, 2010). These circumstances highlight again the need for integrative taxonomy as a tool to better establish species boundaries and to describe new taxa, in order to properly assess their biogeographic and evolutionary history.

Molecular and barcode markers in the study of bryophytes

As mentioned before, the use of DNA sequences in bryophytes studies have increased during the past two decades. Efforts for finding single DNA barcoding markers among plants resulted more difficult than in animals, due to the lower rate of variation in plant plastid DNA (Coissac *et al.,* 2016). Furthermore, the common markers employed in land plants, such as *rbcL* or *matK,* showed also lower resolution for bryophytes species delimitation (Stech & Quandt, 2010; Stech *et al.,* 2013), underlining the need of including the information of a greater number of molecular markers. In the last decade, the available markers have increased, particularly chloroplast ones, and several of them showed high success in phylogenetic analyses: *matK*, *trnH-psbA*, *trnG*, *rps4*, *rbcL*, *trnL-F* and *ITS* (Stech & Quandt, 2010; Liu *et al.,* 2010, 2011), being the last four some of the most employed. Recent studies have shown that these markers might provide sufficient information when comparing also closely related species or for revealing cryptic species complexes for both liverworts and mosses (Medina R. *et al.,* 2012; Renner *et al.,* 2013; Stech *et al.,* 2013; Patiño *et al.,* 2017a), being also informative at intraspecific level for phylogeography and population genetics (Laenen *et al.,* 2011; Pisa *et al.,* 2014, 2015). However, the optimal combination of barcoding markers for bryophytes, especially for species delimitation, is still under discussion.

Furthermore, the reduction of the cost of wide-genome sequencing is making available new molecular techniques. The emergence of Next-Generation Sequencing (NGS) technology and high-throughput sequence determination can improve the efficiency and speed of gene discovery, and will allow more accurate techniques and DNA barcoding for species discrimination and for assessing phylogenetic relationships (for review see Coissac *et al.,* 2016). Within bryophytes, Liu *et al.* (2012) provided new mitochondrial markers suitable for phylogenetic studies by comparing complete mitochondrial genomes of two mosses and testing them with species from other moss lineages. The use of complete genomes has revealed low overall degrees of divergence for the complete organellar genomes and nuclear ribosomal DNA repeat (nrDNA) (Liu *et al.*, 2013), and have also determined the slow rate of evolution of the mitochondrial genome among mosses (Liu *et al.*, 2014). An increasing number of complete mitochondrial and chloroplast genomes from mosses and liverworts have been published in the last years (Bell *et al.,* 2014; Sawicki *et al.*, 2014, 2015; Young-Jun *et al.,* 2015; Myszczyński *et al.,* 2017), allowing partial phylogenetic reconstructions of some mayor bryophytes lineages. Moreover, Lewis *et al.* (2016) recovered the first characterization of infraspecific polymorphism within and across complete mitochondrial and chloroplast genomes and nrDNA of *Tetraplodon fuegianus* Besch, opening the door for the use of wide-genome sequencing for bryophyte studies at intraspecific population level.

Background of the Orthotricheae with focus on *Orthotrichum* and *Lewinskya*

The tribe Orthotricheae Goffinet & Vitt is part of the Orthotrichaceae Arnott, a highly diversified and cosmopolitan family of mosses (Crosby *et al.,* 1999). After several rearrangements of this tribe at the end of the 20th century (Goffinet & Vitt, 1998; Lewinsky-Haapasaari & Hedenäs, 1998; Goffinet *et al.*, 1998), Goffinet & Buck (2004) concluded that the Orthotricheae included four genera: *Orthotrichum* Hedw., *Sehnemobryum* Lewinsky-Haapasaari & Hedenäs, *Stoneobryum* D.H. Norris & H. Rob. and *Ulota* D. Mohr.

From these genera, the most diversified and taxonomically and phylogenetically controversial is probably *Orthotrichum*. It was already included in *Species Muscorum* (Hedwig, 1801), and since then constant taxonomical rearrangements and new species were proposed. These rearrangements are partially originated by the intrinsic complexity of

bryophytes taxonomy, but also to the own complexity of *Orthotrichum*. Plants from this genus commonly display a relative uniform appearance, but they also show a great intraspecific variability of many of their morphological characters that difficult to establish boundaries among different taxa. In the 20th century, Jette Lewinsky and Dale H. Vitt accomplished detailed revisions of the genus for different regions of the world: North America (Vitt, 1973), sub-Saharan Africa (Lewinsky, 1978), Australasia (Lewinsky, 1984a), South America (Lewinsky, 1984b, 1987), or Southeast Asia (Lewinsky, 1992a), with a final worldwide synthesis of Lewinsky (1993). In this latter work, Lewinsky proposed 116 species for the genus, a number that has been increasing during the last decades, up to counting with 168 species in 2016 (Medina R. *et al.*, 2013 updated according to Bosanquet & Lara, 2012; Wang & Jia, 2014; Plasek *et al.*, 2014).

The synthesis works of *Orthotrichum* from Vitt (1971, 1982) and Lewinsky (1993) proposed the classification of the genus organized into subgenera and sections. However, the molecular phylogenetic studies of Goffinet *et al.* (1998) and Goffinet *et al.* (2004) evidenced that *Orthotrichum*, as circumscribed and diagnosed by Lewinsky (1993), was an artificial lineage, since it was resolved as polyphyletic, with cryptoporous and phaneroporous species placed in two different clades within the Orthotricheae. The polyphyly of *Orthotrichum* was repeatedly suggested by following inferences from molecular data (Goffinet *et al.*, 2007; Sawicki *et al.*, 2009; Plášek *et al.*, 2011; Sawicki *et al.*, 2012), although none of these works proposed a rearrangement of the genus, until Sawicki *et al.* (2010) confirmed the results obtained by Goffinet *et al.* (2004) and reinstated *Nyholmiella* Holmen & E. Warncke for *Orthotricum obtusifolium* Brid. and *O. gymnostomum* Bruch ex Brid. Recently, Plášek *et al.* (2015) further proposed a division of *Orthotrichum* by segregating *O. lyellii* Hook. & Taylor in the new genus *Pulvigera* Plášek, Sawicki & Ochyra, and resurrecting *Dorcadion* Adans. ex Lindb. to accommodate the monoicous and phaneroporous taxa of *Orthotrichum*, while the cryptoporous species remained in the genus *Orthotrichum s.str*. However, Lara *et al.* (2016) remarked the illegitimate use of the name *Dorcadion* chosen by Plášek *et al.* (2015), and replaced it by *Lewinskya* F.Lara, Garilleti & Goffinet, together with a subsequent combination of species names and update of their current distributions. Thereby, currently *Orthotrichum s.l.* is segregated into four genera: *Orthotrichum s.str.*, *Lewinskya*, *Nyholmiella* and *Pulvigera*, increasing to seven the number of genera comprising the tribe Orthotricheae.

This thesis is focused on *Orthotrichum s.str.* and *Lewinskya*. As mentioned above, *Orthotrichum* s. l. was a high diversified genus. After its segregation *Orthotrichum s.str.* encloses the highest number of taxa, with a total of 104 (95 species, 1 subspecies and 8 varieties), while *Lewinskya* comprises 70 taxa (66 species and 4 varieties) (Kiebacher & Lüth, 2016; Lara *et al.,* 2016). Both genera have representatives in all continents, growing in a wide variety of habitats, except for deserts of Africa and Asia and lowland tropical areas. Most of the species are epiphytes, although some of them are exclusively or preferentially saxicolous (Lewinsky, 1993; Lara *et al.*, 2016). *Orthotrichum s.str.* and *Lewinskya* are both more diversified along the Northern Hemisphere (83 and 46 taxa respectively), where both harbor higher numbers of endemic taxa (72 and 38 taxa respectively) (Lara *et al.,* 2016). Most of the endemic species are endemic to relatively large areas (i.e. Mediterranean Basin, western or eastern North America, Patagonia), with only few cases of locally restricted endemism, as those of *O. handiense* F. Lara, Garilleti & Mazimpaka (Lara *et al.*, 1999; Patiño *et al.,* 2013), *O. casasianum* F. Lara, Garilleti & Mazimpaka (Mazimpaka *et al.*, 2012), *O. cambrense* Bosanquet & F. Lara (Bosanquet & Lara, 2012) or *L. truncatodentata* (Müll. Hal.) F. Lara, Garilleti & Goffinet (thought to be extinct; Lewinsky, 1992b).

Although most of the species are present in only one continent, both *Orthotrichum* s.tr. and *Lewinskya* encompass species that show wide geographic distributions with intercontinental disjunctions. However, the absence of truly cosmopolitan elements stands out, since the most widespread and disjunct species (i.e. *L. rupestre* (Schleich. ex Schwägr.) F. Lara, Garilleti & Goffinet, *O. diaphanum* Brid., *O. cupulatum* Brid.) are not found in large and important areas of one or both Hemispheres (Lewinsky, 1993). Other types of intercontinental disjunctions are numerous and biogeographically relevant. For example, in the Northern Hemisphere the Eurasian disjunctions are represented by *O. crenulatum* Mitt. (Lara *et al.,* 2010), *O. consobrinum* Cardot (Chinese-Japanese region-Asia Minor-Western Europe) (Lara *et al.,* 2009) or *O. callistomum* Fisch.-Oost. ex Bruch & Schimp. (Eastern Himalayas-Caucasus-Alps) (Lara *et al.,* 2010). In the Southern Hemisphere, different disjunctions have been documented affecting nearly all continents (Lewinsky, 1993), as those of *O. aequatoreum* Mitt. between Central and South America and East Africa, *O. assimile* Müll. between southern South America and Australasia, or *L. firma* (Venturi) F. Lara, Garilleti & Goffinet between East Africa and southwest India.

Especial mention deserves the Holarctic disjunction between North America and Europe, showed by a remarkable number of species of *Orthotrichum* and *Lewinskya* (slightly more than 30% of species). Nevertheless, recent studies demonstrated that this disjunction known for *O. tenellum* (Medina R. *et al.,* 2012) and *O. consimile* (Medina R. *et al.,* 2013) actually hided two complexes of cryptic or pseudo–cryptic species with narrower distributions restricted to each of the sides of the disjunction, and only two of them, *O. pulchellum* Brunt. and *O. columbicum* Mitt., were a true disjunct species. These works, together with one of *Ulota* by Caparrós *et al.* (2016), revealed through integrative approaches that the relative morphological uniformity, but also the considerable intraspecific variation of several traits within species with wide and disjunct geographic ranges, might be obscuring the actual species diversity and distribution patterns within the Orthotricheae. Thereby intercontinental distributions in this tribe, but also in bryophytes, should be carefully revised.

Aims and outline of this thesis

Within the context previously presented, this thesis pursues to answer to different paradigmatic questions of bryology, focusing in the following aims:

- Analyze whether the common broad distribution ranges of bryophytes are truly so and thus imply a real pattern, or instead hide a mosaic of different unknown taxa or cryptic species with narrower distributions.
- Understand the origin of the western Palearctic - western Nearctic disjunction in bryophytes, and the processes shaping this intercontinental distribution pattern.
- Evaluate whether the low endemicity rates of bryophytes are due to dispersal, taxonomical shortcomings or the existence of cryptic species.

These general objectives are addressed through different studies focused in two genera of the Orthotricheae, *Orthotrichum* and *Lewinskya*, from which little is still known about the processes that shape their current evolutionary and distribution patterns, and particularly to several species or complex of species from the Northern Hemisphere.

Thus, other specific objectives necessary to achieve the particular purposes of this research in relation to *Orthotrichum* and *Lewinskya* are addressed:

- Establish species boundaries of the species studied and closely related taxa through an integrative taxonomic approach.
- Evaluate and describe the still unknown diversity within both genera.
- Identify new molecular markers or barcoding regions to perform species delimitation analyses and accomplish phylogeographic studies within both genera, also useful for other Orthotricheae.

These general objectives are dealt with throughout the results chapters of this thesis (3.1 to 3.4), each of them focusing on a different species or complex of species of *Orthotrichum* and *Lewinksya*, with the aim of evaluating whether common patterns of biogeographical processes arise for these two genera that could also be compared to bryophytes in general.

All the studies are presented as scientific papers (chapters 3.1 and 3.2 are already published), and correspond to the results of this thesis. These are preceded by a chapter with a general description of the methodology used in each of them, and followed by the general

discussion and conclusions of this thesis. The following is a brief summary of each of the results chapters.

Chapter 3.1 addresses the study of *Lewinskya acuminata* (H. Philib.) F. Lara, Garilleti & Goffinet[1], a species widespread along the Mediterranean basin and also present in the Canary Islands. Recently, several populations of a moss similar to *L. acuminata* were found in California and Ethiopia, so the first aim of this study is to assess the taxonomic circumscription of those populations and their relationship to *L. acuminata* through an intensive morphological evaluation and the use of molecular data. These analyses reveal that the populations from California and Ethiopia belonged to *L. acuminata*, exposing a case of intercontinental disjunction that is also evaluated through molecular analyses.

Chapter 3.2 [1] aims finding new variable molecular markers for species delimitation and phylogeographic studies comparing the complete mitochondrial genome of two *Orthotrichum* species. First, two genome sequences of *O. diaphanum* are compared with one sequence of *O. macrocephalum* for searching new interspecific variable regions useful for phylogenetic analyses and species delimitation. Second, both sequences of *O. diaphanum* are analyzed to look for intraspecific variable markers suitable for phylogeographic and population studies within Orthotricheae. Finally, a phylogenetic analysis of the Orthotricheae is done with the obtained sequences and other published mitochondrial genomes to evaluate the congruence of the known relationships of *Orthotrichum s.l.* and *Ulota*, and the polyphyletic nature of *Orthotrichum s.l.* previously obtained with a reduced number of molecular markers.

Chapter 3.3 focuses in a California-Macaronesia disjunction within *Orthotrichum s.str.* Populations of a new moss were found in Tenerife Island (Canary Islands), resembling two endemic Californian species: *Orthotrichum shevockii* Lewinsky-Haapasaari & D.H. Norris and *O. kellmanii* D.H. Norris, Shevock & Goffinet. Morphological and molecular analyses are implemented to assess the species boundaries of those two similar taxa and whether the new populations of Tenerife can be circumscribed to any of them or consist on a new species.

[1] Since chapter 3.1 and 3.2 were published previously to the current proposed division of genus *Orthotrichum s.l.* in two genera, *Orthotrichum s.str.* and *Lewinskya* (Lara *et al.*, 2016), the previous species names are maintained in both chapters although in the rest of this memory we refer to them with their current proposed names.

Besides, the evolutionary relationships of the implicated taxa and the origin of this disjunction are evaluated with molecular dating and ancestral area estimation analyses.

Chapter 3.4 is centered in the study of *Lewinskya affinis* (Brid.) F. Lara, Garilleti & Goffinet, a widespread species in the Holarctic, showing an intercontinental disjunct distribution among Europe, Macaronesia, North and East Africa, southwest Asia and North America. Several authors have pointed out a remarkable variation of certain characters along its distribution, and others have suggested the possible existence of different taxa within *L. affinis* as response to that variation. A species delimitation study using different molecular and morphological analyses is performed to establish whether *L. affinis* is actually a species with a disjunct distribution or a complex of species with narrower distributions.

Chapter

2

Materials and methods

This section includes general aspects of the methodology used along the different chapters of this thesis. Detailed taxon sampling, specific methods and software used, details of each statistical or molecular analysis and other particular information can be found in the corresponding "material and methods" section of each chapter.

Taxon sampling includes specimens collected in different *ex professo* field campaigns throughout Europe, Canary Islands, North and East Africa, and western North America by the author of this thesis and others members of the Spanish research group on Orthotrichaceae. It also comprises specimens from the herbaria of the Universidad Autónoma de Madrid (MAUAM) and the Universidad de Valencia (VAL) obtained during previous collecting campaigns of the same research group. The material examined has been completed with loans from other herbaria: BM, CAS, CONN, M, MUB, NY, OP, PC, TFC, and UC. All the species have been sampled based on material availability and intending to represent their distribution range and morphological diversity. The list of studied materials and GenBank accession numbers are given as an appendix at the end of each chapter.

The methodology of chapters 3.1, 3.3 and 3.4 corresponds to an integrative approach. The three studies first evaluate the circumscription and delimitation of the studied species, in each case using different types of analyses of morphological and molecular data. Once the species boundaries are established, biogeographic hypotheses are tested through molecular based phylogenies. These phylogenies have been complemented with dating analyses and ancestral area estimations, when possible. Chapter 3.2 is fundamentally a molecular methodological work and will be treated only in the section regarding DNA and phylogenetic analyses section of this chapter.

Morphological analyses

All the studied specimens have been examined under both stereo and light microscopes. In the first instance, plant height was measured in dry specimens using a calibrated ruler. After that, one shoot was randomly selected from one tuft, and analyzed to evaluate the remaining characters. Qualitative traits analyzed in dry conditions were first examined using a stereo microscope, and then each shoot was mounted separately on a glass slide with water and fixed later with glycerogelatin to investigate the rest of morphological characters. Basal

and mid vegetative leaves and perichaetial ones were mounted separately on the slide to avoid possible confusion, since in some species each group of leaves can show differences for some morphological traits, but in others they can be relatively similar. Microscopic measures were taken using the calibrated ruler included in the ocular of the light microscope, or with the measurement tool of the digital software Olympus LabSense 1.1 associated to an Olympus UC30 digital camera. All the specimen images taken under the stereo microscope are digitally stacked photomicrographic composites of up to 30 individual focal planes obtained using the software package Zerene Stacker 1.04. SEM microphotographs were taken from air-dried, gold-sputtered samples at University of Valencia.

In all cases, morphological analyses included qualitative and quantitative traits that had been selected based on the experience of the research group on the taxonomy of the Orthotricheae (e.g., Medina R. *et al.*, 2012, 2013; Lara & Garilleti, 2014; Lara *et al.*, 2016), as well as on the literature cited for each of the species. Figure 3.1.2 shows some of the representative traits considered and where they have been measured. The specific characters evaluated for each species can be found in the corresponding chapter. Five replicates per individual were taken for each character to evaluate the within-plant phenotypic variation. For plant height, the length was measured on five plants per specimen, and one individual shoot was randomly selected for taking the rest of the measurements. Due to the common scarcity of well-preserved capsules, the sporophytic traits were measured for one to five capsules, depending on the number of capsules available per specimen. Means from replicates were then calculated to construct the final data set used for statistical analyses.

Multivariate analyses of quantitative traits were performed in chapters 3.1, 3.3 and 3.4, and also for qualitative traits in chapter 3.4. Principal component analyses (PCA) were used to explore the existence of an apparently unknown underlying structure within the datasets that could reflect morphological differences between geographical regions or indicate the possible existence of different morphospecies. A discriminant function analysis (DFA) was also performed to test the statistical support of the predefined geographical groups (chapter 3.1) or those groups identified with molecular and morphological analyses (chapter 3.4), followed by a cross-validation analysis to test the predicting classification power of the DFA analysis. To test for differences in each quantitative morphological trait between the recovered groups, a post-hoc test of multiple comparisons of group means was performed. Descriptive statistics for quantitative traits were also computed and summarized in form of

beanplot graphs (Kampstra, 2008) using the original morphological information considering all replicates. Morphological statistic analyses were performed with SPSS v.22 (IBM Corp., 2013) and the free software R (R Core Team, 2013).

Molecular analyses

DNA extraction, amplification and sequencing

DNA extraction, amplification, purification and sequencing of material for molecular analyses was mainly performed in Universidad Autónoma de Madrid (materials for chapters 3.1 and 3.3, and part of those included in chapter 3.4), but also in two foreign research centers: University of Liege (the rest of chapter 3.4), and University of Connecticut (materials for chapter 3.2). Chapter 3.2 has its own specific molecular protocols that are detailed in the corresponding section. For general information about genome DNA extraction, amplification and sequencing see Liu *et al.* (2014).

Within *Orthotrichum* and *Lewinskya*, it is usual that two or more different species grow intermingled forming mixed tufts, even from both genera. Moreover, the lack of sporophytes generally makes difficult to discriminate species. Taking this into account, in chapters 3.1, 3.3 and 3.4, DNA was extracted from one single individual shoot selected from each specimen, always bearing sporophyte, and using only the upper part of the stem and branches. The discarded sporophyte and the rest of the gametophyte were mounted on glycerogelatin on a glass slide to make possible the further checking of the identification of the materials. DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions, except for several samples extracted at University of Liege using the standard CTAB protocol (Doyle & Doyle, 1987). For chapters 3.1 and 3.3 different molecular markers previously used in other bryophytes studies at inter- and intraspecific level were tested (Stech & Quandt, 2010; Laenen *et al.*, 2011; Pisa *et al.*, 2013), with focus on those included in recent works of genus *Orthotrichum s.l.* (Sawicki *et al.*, 2009; Medina R. *et al.*, 2012, 2013). Some of them showed problems for amplification or sequencing (i.e. nuclear ITS1, *AdK*), and others exhibited low variation at intraspecific level or within the group of species analyzed (i.e. *rpl*16, *trnG*), and were thus discarded. The final selection of molecular markers includes three chloroplast loci, namely

atpB-rbcL, *rps*4, and *trnL-F*, and two nuclear regions namely ITS2 and *Ort*-LFY. Due to the low resolution of the chloroplast and nuclear loci used in these chapters, in chapter 3.4 the chloroplast *rps*4 was used, together with three different loci already tested with *L. affinis* by the research group of University of Liege: *rpl*32-*trnL*$^{(UAG)}$ from the chloroplast genome and two nuclear expressed sequence tag (EST) from the work of McDaniel *et al.* (2013). These new markers showed higher levels of variation, especially both EST regions, although they were difficult to amplify, and they could not be sequenced for all the selected samples.

Primer information of each loci and PCR amplification protocols are found in each chapter. In all cases, the PCR was performed using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech Inc) in a final reaction volume of 25 μL according to the manufacturer's instructions, with 2-4 μL of template DNA for each locus (depending on the quality and concentration of the template), except for both ETS regions where 5-10 μL of template DNA were used. PCR products were visualized in a 1% agarose gel and then purified using Exo/SAP protocol (Thermo Fisher Scientific, Spain). Samples were incubated with 1 μL of Exo1 enzyme and 4 μL of FastAP following the manufacturer's instructions. Cleaned PCR products were sequenced by Macrogen (www.macrogen.com).

Phylogenetic analyses

In chapters 3.1, 3.3, and 3.4, for each DNA region, forward (5'–3') and reverse (3'–5') sequences were edited and assembled into contigs using Geneious 9.0.2 (http://www.geneious.com; Kearse *et al.*, 2012) or PhyDE v.0.9971 (Müller *et al.*, 2006). Sequences were trimmed at both ends and aligned using the software MUSCLE (Edgar, 2004), and finally edited manually with PhyDE or Geneious, inserting gaps where necessary to preserve positional homology. The final sequence matrix of each region was analyzed, when necessary, with GBlocks (Talavera & Castresana, 2007) to identify ambiguous or incomplete regions of data, in order to exclude them from subsequent analyses.

Phylogenetic inferences were based on maximum parsimony (MP, chapter 3.1), maximum likelihood (ML, chapters 3.2 to 3.4) and Bayesian inference (BI, all chapters) analyses. The best-fitting substitution models for each locus were inferred under the Bayesian Information Criterion (BIC) in jModelTest v.2.1.3 (Darriba *et al.*, 2012). Indels of each matrix were coded as informative in an adjacent block with the program SeqState

(Müller, 2012) using the simple indel coding method (Simmons & Ochoterena, 2000). The analyses were performed with and without codified indels with the same parameters indicated above, using model F81 for the indel partition as recommended by Ronquist *et al.* (2011), and the results with better resolution and support for principal nodes were selected for further analyses. All loci data sets were combined in a single concatenated matrix, after visually checking the congruence of independent analyses for each locus in branches with high support (PP $\geq$ 0.95 and BS $\geq$ 85), especially those affecting at species delimitation level. The resulting concatenated data set was analyzed in PartitionFinder (Lanfear *et al.*, 2012) to select the best partitioning scheme and corresponding substitution model for each analysis, using the greedy algorithm with linked branch lengths under the BIC criterion.

Maximum parsimony analyses were performed only in chapter 3.1, using the program TNT 1.0 (Goloboff *et al.*, 2003), with the tree bisection reconnection (TBR) swapping algorithm. Non-parametric bootstrapping (BS) was obtained using the default settings in TNT, with replicates set to 1000. Maximum likelihood analyses were run with RAxML 8 (Stamatakis, 2014), and the best ML tree was selected from 100 iterations and its support was assessed with 1000 replicates of bootstrap resampling under the ML criterion. Bayesian inference phylogenetic analyses were carried out using MrBayes v.3.2.1 (Ronquist *et al.*, 2012) with the substitution models and partitions defined for each case. The number of generations of each analysis depended on the time taken by the four MCMC chains to reach the convergence, and wheter independent loci or the combined matrix was analyzed, varying between 2 and 5 million of generations. Posterior probabilities (PP) were estimated from the 50% majority-rule consensus trees after a burn-in of 25% of the starting trees. The rest of details for each of the different analyses are presented in the corresponding chapters. The resulting trees for all analyses were plotted using FigTree v.1.4.2 (Rambout, 2012).

In chapter 3.4, two different molecular analyses for species delimitation were also performed. The General Mixed Yule-Coalescent (GMYC) model was used for species delimitation following three implementations, the single (sGMYC; Pons *et al.*, 2006) or multiple thresholds (mGMYC; Fujisawa & Barraclough, 2013) methods, based on a likelihood approach, and the Bayesian version of the GMYC model (bGMYC; Reid & Carstens, 2012). For validation analyses of the different species delimitation hypotheses formulated, *BEAST species trees were inferred for each hypothesis using *BEAST v.1.8.0 (Drummond *et al.*, 2012), and a Bayes Factor Delimitation analyses was performed to assess

the hypothesis that best fit the data following the framework proposed by Grummer *et al.* (2014) and Hotaling *et al.* (2016). Both analyses were performed on an ultrametric tree previously obtained with BEAST, and details for both of them are presented in the corresponding section.

In chapter 3.2, only protein-coding genes were used for phylogenetic analyses of the mithochondrial genomes. The obtained sequences were aligned using the progressive Mauve algorithm (Darling *et al.,* 2004) in Geneious, and manually edited. Phylogenetic analyses were performed under ML and BI. Maximum-likelihood analyses were executed using the parallel version of RAxML v.8 (Stamatakis, 2014). The ML trees were calculated under the GTR+G model. Nonparametric bootstrap analyses were implemented by GTR–CAT approximation for 100 pseudo-replicates. Bayesian inference was conducted using MrBayes v.3.2 (Ronquist *et al.*, 2012). The analysis was performed with two runs, each having four chains. Markov Chain Monte Carlo (MCMC) was run for 10 million generations, with trees and parameters sampled every 1000 generation.

Biogeographical analyses

In chapter 3.3, biogeographic inferences were performed including divergence time estimation and ancestral areas reconstruction analyses. Details for both analyses are presented in the corresponding section. Divergence times were estimated using BEAST 1.8.0 (Drummond *et al.,* 2012), testing both strict and uncorrelated log-normal relaxed clocks under two different speciation tree models: Yule and birth–death process. In the absence of fossil records of *Orthotrichum*, an absolute nucleotide substitution rate (mean = 4.453E-4 and stdev = 1.773E-6 substitutions/site/millions of years) was used and sampled from a log-normal distribution according to the results of relaxed-clock analyses across mosses (Laenen *et al.,* 2014). The best model was selected through Marginal likelihood estimation (MLE) assessed using path-sampling (PS) and stepping-stone (SS) methods. The resulting tree was summarized in TreeAnnotator 1.7.5 (Drummond *et al.,* 2012) and viewed in FigTree v.1.4.2. Results based on nucleotide substitution rates should be interpreted with caution since these rates can considerably vary among lineages and between different DNA markers (Laenen *et al.,* 2014; Villarreal & Renner, 2014).

For ancestral area estimation, geographical areas were defined considering the distribution range of all taxa considered in the ingroup. The time-calibrated tree obtained from BEAST, without outgroups, was used to perform ancestral-area estimations with the R package BioGeoBEARS (Matzke, 2014). Six different biogeographical models (DEC, DEC + J, DIVALIKE, DIVALIKE + J, BAYAREA, BAYAREA + J) were applied under a maximum likelihood framework, and compared using the Akaike Information Criterion (AIC) (Matzke, 2013, 2014).

Chapter

3

Results

Chapter

3.1

Lewinskya acuminata

Is it really you, *Orthotrichum acuminatum*? Ascertaining a new case of intercontinental disjunction in mosses.

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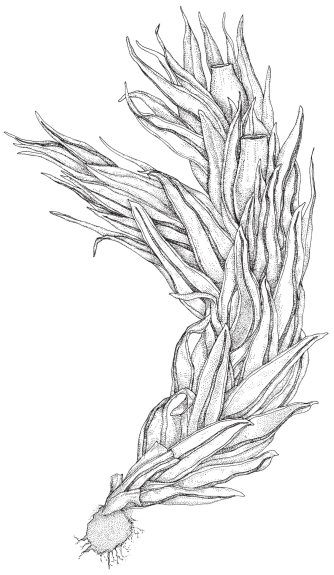
Published in *Botanical Journal of the Linnean Society* (2016), 180: 30–49



Abstract

Intercontinental disjunct distributions are a main issue in current Biogeography. Bryophytes usually display broad distribution ranges and therefore constitute an interesting subject of study in this context. Through the course of recent field work in Western North America and Eastern Africa, we found new populations of a moss morphologically very similar to *Orthotrichum acuminatum*. So far this species has been considered to be one of the most typical epiphytic mosses of the Mediterranean Basin. The new findings raise some puzzling questions: Do these new populations belong to cryptic species, or do they belong to *O. acuminatum*, a species which then has a multiple-continent disjunct range? In the latter case, how could such an intercontinental disjunction be explained? To answer these questions, an integrative study involving morphological and molecular approaches was conducted. Morphological results reveal that Californian and Ethiopian samples fall within the variability range of those from the Mediterranean Basin. Similarly, the phylogenetic analyses confirm the monophyly of these populations, evidencing that *O. acuminatum* is one of the few moss species whose distribution range comprises the Western Nearctic, the Western Palearctic and Eastern Africa (Paleotropical). Pending a further genetic and phylogeographic study to support or reject the hypothesis, a process of long distance dispersal (LDD) is hypothesized to explain this distribution, and the origin of the species is suggested to be the Mediterranean Basin, from where diaspores of the species may have moved to California and Ethiopia. The spore release process in *O. acuminatum* is revisited to support the LDD hypothesis.

Introduction

Bryophytes usually display broad distribution ranges that often span across different continents (Schofield & Crum, 1972; Schofield, 1988; Medina N.G. *et al.,* 2011). Their dispersal capabilities are in general high, since they can produce huge amounts of microscopic diaspores (Schofield, 1992; Frahm, 2008). Due to the small size of spores, wind may act as the main transport vector (Wilkinson *et al.,* 2012), although dispersal by water and animals has also been reported (Marino *et al.,* 2009; Hutsemékers *et al.,* 2013; Lewis *et al.,* 2014). However, not only spore size and type are critical for bryophytes dispersal, but also different factors affect the capabilities of these organisms to expand their areas, which may lead to differences in their geographic ranges. These factors include accessibility to transport vectors, survival to harsh conditions during and after their transport, establishment success and persistence (van Zanten & Pócs, 1981; Medina N.G. *et al.,* 2011). Spore release mechanisms, including those related with xero- or hygrocastique release (Lazarenko, 1957), are also a key factor. Significant intercontinental links among the bryophyte floras have traditionally supported the hypothesis that current disjunctions are the result of the fragmentation from a wide ancient distribution (Schofield & Crum, 1972; Schofield, 1988). This idea is reinforced by the parallelism displayed by similar disjunctions of seed plants (but see Christenhusz & Chase, 2013). However, while disjunctions in seed plants involve different species or genera, bryophyte disjunctions mostly affect populations within the same species (Shaw, 2001). Currently, different molecular studies state opposite evolutionary histories for diverse cases of disjunctions in bryophytes. Studies such as those by McDaniel & Shaw (2003), Heinrichs *et al.,* (2006), and Hedenäs (2008) support the hypothesis of ancient fragmentation for particular species, whereas other works state long distance dispersal (LDD) as the origin of different disjunction patterns (Muñoz *et al.,* 2004;

Vanderpoorten *et al.,* 2008; Piñeiro *et al.,* 2012; Shaw *et al.,* 2013), including that of the Mediterranean-Western North America or, more comprehensively, Western Palearctic-Western Nearctic (Shaw *et al.,* 2003; Huttunen *et al.,* 2008).

Complementarily, molecular approaches to the study of intercontinental disjunctions in bryophytes reveal not only that not all of them have the same biogeographic history, but also that not all those traditionally considered to be disjunct are actually so. The origin of these misperceptions could be either taxonomic confusions (Renner *et al.,* 2013; Hedenäs *et al.,* 2014) or the existence of cryptic species (Shaw, 2001; Heinrichs *et al.,* 2009). Integration of morphological and molecular analyses has demonstrated to be an effective way to evaluate legitimate disjunctions in bryophytes. Within the genus *Orthotrichum* Hedw., recent integrative taxonomic studies on European-Western North American supposedly disjunct species have revealed, in most cases, the existence of several morphologically distinct species instead of disjunct populations of the same species (Medina R. *et al.,* 2012, 2013). Most of the resulting species are restricted to one or other of the regions involved in the disjunction, so that the resulting pattern is instead a complex of continental endemics. Conversely, true disjunctions have been confirmed for a few of the species traditionally considered disjunct (Medina R. *et al.,* 2012).

This work deals with *Orthotrichum acuminatum* H. Philib., a moss currently considered to be restricted to the Western Palearctic area (Lara & Garilleti, 2014). It is an epiphytic moss frequent in most of the islands and continental countries bordering the Mediterranean Sea (Draper *et al.,* 2006; Draper *et al.,* 2008; Ros *et al.,* 2013) and the Canary Islands (Lara *et al.,* 1999; González-Mancebo *et al.,* 2009). Because of this wide but environmental-specific geographic range, it has been considered one of the best examples of Mediterranean moss distribution (Lara & Mazimpaka, 2001; Mateo *et al.,* 2013). However, it is not strictly an endemism of this biogeographic area, since it has also been reported in non-Mediterranean Europe, namely in The Netherlands (van der Pluijm, 2001), Germany (Ahrens, 2004; Meinunger *et al.,* 2007) and Great Britain (Blockeel, 2009). Most of these extra-Mediterranean finds of *O. acuminatum* correspond to meager populations and could represent cases of transient populations (van der Pluijm, 2001; Blockeel, 2009).

As a result of the bryological surveys carried out by our team in different parts of the world, new populations that could correspond to *Orthotrichum acuminatum* have been

discovered in Western North America and Eastern Africa. Interestingly, this moss shows morpho-functional sporophytic traits related to hygrocastique spore release that could be interpreted as adaptations to a specific environment, and thus could limit the efficiency in spore spreading, thereby reducing its capabilities for LDD (see below). The fact of having a wide Mediterranean distribution despite its apparently limited spore dispersal, together with the new putative populations found in North America and Africa, make this moss an interesting target for studies on intercontinental disjunctions.

Orthotrichum acuminatum is easily recognizable by its long-acuminate perichaetial leaves and its characteristic fusiform capsules with reduced peristome. Capsules are almost smooth with superficial stomata, a puckered mouth when dry, constricted by eight short and moderately marked ribs, and a suboral ring of greenish to brownish coloured cells. When present, the exostome is rudimentary, whereas the endostome segments are always well developed and stout, uni- or biseriate with strong papillose ornamentation. The mentioned sporophytic traits are related to the hygrocastique spore release of *O. acuminatum* (Lara *et al.*, 1999). The capsule mouth remains puckered when dry, with the broad incurved endostome segments impeding spore release. When moist, the exothecium expands and the segments become separated and somewhat erect, allowing spore liberation. Spores are subsequently released during high humidity periods which probably allows rapid germination. This could be advantageous either when favorable environment conditions are ephemeral or when the spores are intolerant to water stress periods, a situation that recalls the endosporic germination described in other epiphytic bryophytes (Allen, 1987; Schuette & Renzaglia, 2010; Garilleti *et al.*, 2012). However, hygrocastique spore release may negatively affect spore dispersal, since wet conditions increase spore weight, aggregation and deposition near their original capsule (Mueller & Neumann, 1988). This particular type of spore release is exceptional in the genus, only occurring in a small number of epiphytic species mostly confined to Mediterranean climate areas of the world, like *O. acuminatum* (Lara & Mazimpaka, 1993; Garilleti, *et al.*, 2006; Lara *et al.*, 2009; Garilleti *et al.*, 2011).

Within its Mediterranean range, *Orthotrichum acuminatum* grows under a wide range of temperatures and precipitations, being only absent in the most arid situations. Although it is more frequent in mountain areas, its altitudinal range varies from (0-)150 to 1850(-2100) m.a.s.l. (Draper *et al.*, 2006; Lara & Garilleti, 2014). Consequently, it lives in a wide variety of Mediterranean scrubland and forest communities dominated by either deciduous or

evergreen species. In Western North America, the recently discovered populations of the *O. acuminatum*-like moss are confined to San Jacinto and San Bernardino mountains in the southernmost part of California. There, the moss grows between 1200 and 1500 m.a.s.l. in forests dominated by *Quercus* spp., occasionally accompanied by *Pinus ponderosa* P. Lawson & C. Lawson. This area of Southern California is subject to a Mediterranean climate that matches the environmental affinities of *O. acuminatum* in the Mediterranean Basin. In eastern Africa, the findings consist of two nearby populations from the volcanic area of Simien Mountains in northern Ethiopia. The moss was found there at an altitude of around 3500 m.a.s.l. on trunks of tree-like heather *Erica trimera* (Engl.) Beentje, on the slopes of two different valleys dominated by ericaceous vegetation with scattered *Hypericum revolutum* Vahl and *Lobelia rhynchopetalum* (Hochst. ex A. Rich.) Hemsl. Ethiopian afro-montane ericaceous woodlands are the dominant vegetation of the high elevation areas in the region, and develop under an afro-alpine climate characterized by small seasonal temperature variations, but large diurnal temperature oscillations throughout the year. This situation could imply ecological conditions fairly different from those prevailing in the typical Mediterranean environment where populations of *O. acuminatum* mostly grow, but a number of other plant species thrive under both types of climates (e.g. Desamoré *et al.*, 2011).

The discovery of these new populations in distant geographic areas raises the question of whether *Orthotrichum acuminatum* is a widespread species –with a multiple-continent disjunct range instead of an area restricted to the Western Palearctic–, or a complex of cryptic species distributed in different continents. Assuming the LDD limitation due to its type of spore release (hygrocastique), our expectation would be the cryptic species scenario. To test this hypothesis, we have studied previously known and new-found putative populations of *O. acuminatum* through an integrative taxonomic approach based on morphological and morphometric analyses combined with phylogenetic inferences of DNA sequences. The specific objectives of this study are to: 1) evaluate the morphological and genetic variation in *O. acuminatum* throughout its distribution range, searching for possible distinctiveness that could reveal the existence of different taxa or, contrarily, substantiate the monophyly of *O. acuminatum*; and 2) to understand the underlying biogeographic processes that may have led to such a distribution pattern.

Material and methods

Sampling design

The studied specimens were selected in order to represent the whole distribution and ecological range of the species (Fig. 3.1.1 and Appendix). Seventy-four samples were included: 56 from the Mediterranean Basin, eight from Ethiopia and ten from California. Fifteen of these samples were used for the molecular analyses: eight from the Mediterranean Basin, three from Ethiopia and four from California (Fig. 3.1.1). Twelve specimens of six other *Orthotrichum* species with superficial stomata were included to provide a phylogenetic frame to assess the monophyly of *Orthotrichum acuminatum*. One specimen of *Macrocoma* (Hornsch. ex Müll.Hal.) Grout and two of *Zygodon* Hook. & Taylor were selected as outgroup (Goffinet *et al.*, 2004). Thirty samples representing 11 taxa were thus included in the molecular study. Studied material is kept at MAUAM, MUB and VAL (see Appendix).

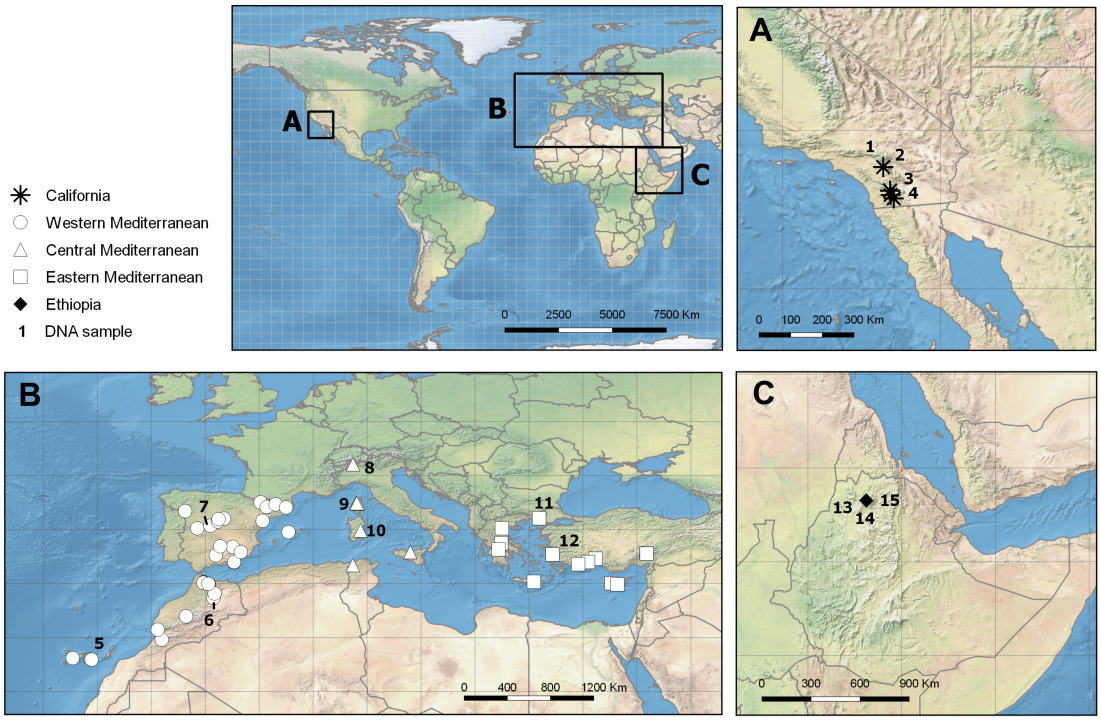


Figure 3.1.1. Geographic distribution of the studied specimens of *Orthotrichum acuminatum*. Symbols indicate specimens included in the morphometric analyses according to the established geographic areas. Numbers indicate specimens included both in morphometric and phylogenetic analyses (see Appendix).

Morphological analyses

An intensive morphometric analysis was conducted to highlight whether morphological differences actually occurred between the disjunct regions. Eighty-seven morphological characters (60 qualitative and 27 quantitative; Tables 3.1.1 and 3.1.S1) were selected on the basis of our own experience in previous studies on Orthotrichaceae (Lara *et al.,* 2009; Medina R. *et al.,* 2009, 2012, 2013; Lara & Garilleti, 2014). Some of the representative traits considered and where they have been measured are shown in Figure 3.1.2. Leaf cell sizes were taken from upper non-perichaetial leaves, in each case measuring length and width from the same cell, including the cell wall (Fig. 3.1.2A). The length of leaf acumen is that from the tip of the leaf to the point where the margin begins to recurve, taken in the side where this length is shorter (Fig. 3.1.2B). The so-called suboral ring corresponds to the zone just below capsule mouth, and is formed by short, thick-walled, coloured cells (Fig 2C, 2D). Five replicates were taken for each character to evaluate the within-plant phenotypic variation. For plant size, the length was measured on five plants per specimen, and one of them was selected for taking the rest of the measurements. Due to the common scarcity of well-preserved capsules, the sporophytic traits were measured for one to five capsules, depending on the number of capsules available per specimen. Means from replicates were then calculated to construct the data set used for the statistical analyses. As a result, two morphological matrices were analysed, the qualitative one with 74 specimens and 60 variables, and the quantitative one with 74 specimens and 27 variables.

To explore the possible existence of geographic patterns related to the morphological variation within *Orthotrichum acuminatum*, each sample was classified prior to the statistical analyses in each of the following three main geographical regions: Mediterranean Basin (N = 56), California (N = 10) and Ethiopia (N = 8). Furthermore, the Mediterranean Basin is subdivided in three sub-regions: Western Mediterranean including Canary Islands (N = 32), Central Mediterranean (N = 10) and Eastern Mediterranean (N = 14). Descriptive statistics (mean, standard deviation and ranges) were computed for all quantitative variables for each of the five geographical groups. Univariate analysis of variance (ANOVA) was conducted to assess the homogeneity of variances for each of the 27 quantitative variables. Multivariate analysis of variance (MANOVA) was performed using the 27 variables and the five geographical groups as the levels for the factor. To test for differences between geographical groups, a post-hoc test of multiple comparisons of group means with Unequal N was

performed. To explore for unknown underlying structure within the dataset an exploratory multivariate analysis was performed (principal component analysis, PCA). Only principal components (PCs) accounting for more than 10% of the variance were considered in the results. A discriminant function analysis (DFA) was performed to test the statistical support of the predefined geographic groups. The DFA was first run according to the three main geographic regions, and also with five groups to evaluate the possible relationship of California or Ethiopia with a specific sub-region within the Mediterranean (Western, Central or Eastern). DFA was computed using a cross-validation approach and the prior probabilities according to group size. Multivariate analyses were run with missing values and also replacing them by the mean value of each character. Results from both approaches were congruent (results not shown). We used the data set with missing values replaced by the mean for the final analyses to avoid sampling decrease. For MANOVA, the type III sum of squares was selected. A correlation matrix was used in the PCA to scale the morphological variables.

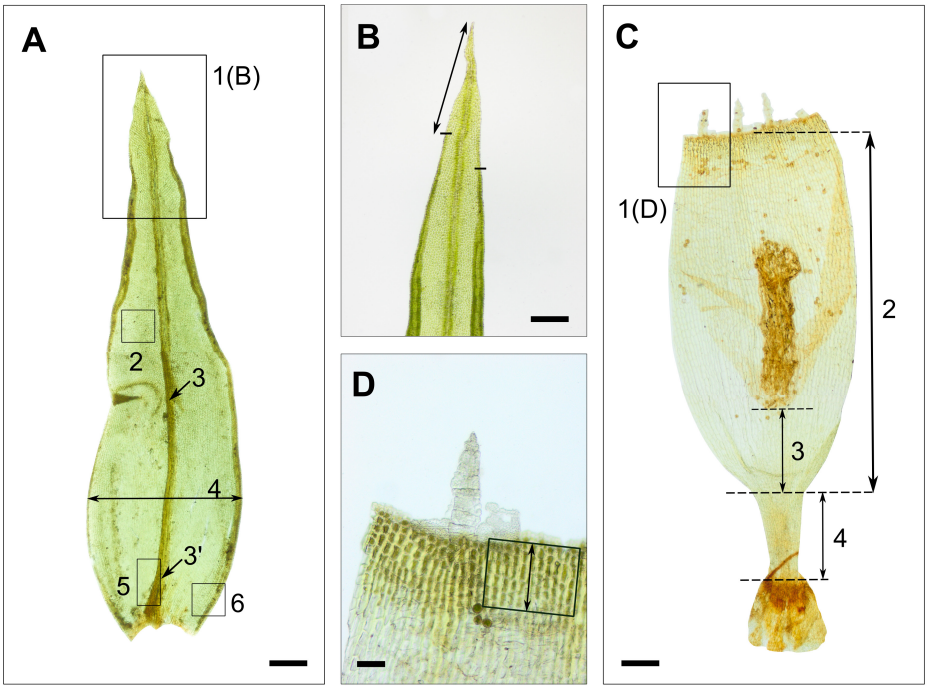


Figure 3.1.2. Measurement explanation of some quantitative traits. A: Leaf quantitative traits: 1= length of leaf acumen (see B for details), 2 = length and width of cells from leaf lamina, 3 = costa width at central lamina, 3' = costa width at leaf base, 4 = maximum width of leaf, 5 = length and width of paracostal cells, 6 = length and width of marginal cells; B: length of leaf acumen, bars indicate the point where margins become recurved; C: capsule quantitative traits: 1 = suboral ring length (see D for details), 2 = capsule length, 3 = length of capsule neck, 4 = seta length. D: capsule suboral ring length, the rectangle defines a uniform area between exothecial bands where this length was measured. A-B: California MAUAM-Brio 3318, C: Mediterranean, Spain, MAUAM Brio 3155, D: Ethiopia MAUAM-Brio 3308. Scale bars: A-B, C = 200 µm, D = 50 µm.

Table 3.1.1. Quantitative characters analysed and results for quantitative morphometric analyses for specimens of *Orthotrichum acuminatum*.

Character	*Orthotrichum acuminatum*	California	Western Mediterranean	Central Mediterranean	Eastern Mediterranean	Ethiopia	ANOVA	PC1	PC2
Gametophyte									
Shoot length [1]	1.0 ± 0.3 [0.5-2]	1.0 ± 0.1 (a)	1.1 ± 0.3 (a)	1.0 ± 0.1 (a)	0.9 ± 0.2 (a)	1.2 ± 0.2 (a)	2.588 *	0,028	**-0,292**
Lower leaf length [2]	2.6 ± 0.4 [1.9-3.4]	3.0 ± 0.2 (a)	2.7 ± 0.4 (ab)	2.6 ± 0.5 (ab)	2.5 ± 0.4 (b)	2.4 ± 0.2 (b)	3.526 *	**0,328**	-0,011
Lower leaf width [2]	0.7 ± 0.1 [0.4-0.9]	0.8 ± 0.1 (a)	0.7 ± 0.1 (ab)	0.7 ± 0.1 (ab)	0.7 ± 0.1 (b)	0.6 ± 0 (b)	3.128 *	**0,311**	0,034
Upper leaf length [2]	3.5 ± 0.4 [2.5-4.6]	3.8 ± 0.3 (a)	3.6 ± 0.3 (ab)	3.3 ± 0.4 (b)	3.4 ± 0.4 (b)	3.4 ± 0.3 (ab)	4.326 **	**0,324**	-0,097
Upper leaf width [2]	0.9 ± 0.1 [0.7-1.2]	1.0 ± 0.1 (a)	0.9 ± 0.1 (ab)	0.9 ± 0.1 (abc)	0.9 ± 0.1 (bc)	0.8 ± 0.1 (c)	4.487 **	**0,334**	0,096
Upper leaf acumen length	420.2 ± 69 [294-602]	443.3 ± 46.5 (a)	416.2 ± 63.7 (ab)	416.3 ± 57.2 (bc)	365.3 ± 54.2 (bc)	508.4 ± 58.2 (c)	8.057 ***	0,041	**-0,374**
Perichaetial leaf length [2]	4.5 ± 0.4 [3.6-5.4]	4.9 ± 0.3 (a)	4.6 ± 0.4 (ab)	4.3 ± 0.4 (ab)	4.1 ± 0.3 (bc)	4.7 ± 0.3 (c)	8.518 ***	0,195	**-0,355**
Perichaetial leaf width [2]	1.1 ± 0.1 [0.7-1.3]	1.2 ± 0.1 (a)	1.1 ± 0.1 (ab)	1.0 ± 0.1 (b)	1.0 ± 0.1 (b)	1.1 ± 0.1 (ab)	4.410 **	0,168	-0,192
Perichaetial leaf acumen length	620.9 ± 129.9 [386-914]	649.9 ± 86.5 (ab)	661.4 ± 132 (ab)	587.9 ± 140 (ab)	540.4 ± 138.4 (b)	604.7 ± 70.8 (ab)	2.660 *	0,111	-0,244
Costa width at base	67.9 ± 7.4 [51-102.5]	65.1 ± 5.2 (ab)	68.3 ± 5.3 (ab)	73 ± 12.1 (a)	69.5 ± 6 (a)	60.4 ± 6.6 (b)	4.409 **	0,164	0,217
Costa width at central lamina	64.4 ± 6.5 [50-87.5]	64.1 ± 6.2	65.4 ± 5.3	66.1 ± 9.7	65 ± 5.5	58.2 ± 5.3	2.426	0,224	0,224
Upper cell length	16 ± 1.5 [12.8-21.7]	16.8 ± 0.7 (a)	16.4 ± 1.4 (ab)	15.4 ± 1.2 (ab)	15.8 ± 1.8 (ab)	14.4 ± 1.3 (b)	5.018 **	0,219	0,056
Upper cell width	11.2 ± 0.8 [9.5-13.5]	11.9 ± 0.6 (a)	11.3 ± 0.9 (ab)	10.8 ± 0.6 (b)	11.2 ± 0.8 (ab)	10.6 ± 0.5 (b)	4.277 **	0,145	0,026
Paracostal cell length	47.1 ± 6.5 [30-66.9]	49.5 ± 4.6	47.6 ± 7.1	47.2 ± 3.5	46.4 ± 8.3	43.4 ± 4.6	1.08	0,210	0,107
Paracostal cell width	10.7 ± 0.9 [9-13.8]	11.7 ± 0.9 (a)	10.8 ± 0.9 (b)	10.5 ± 0.6 (b)	10.2 ± 0.7 (b)	10 ± 0.4 (b)	7.814 ***	0,262	0,100
Marginal cell length	16 ± 1.2 [12.5-18.8]	16.6 ± 1.2	16 ± 1	16 ± 1.3	15.8 ± 1.5	15.3 ± 1.4	1.332	0,211	0,089
Marginal cell width	12.4 ± 1 [9.5-14.8]	13.1 ± 0.8 (a)	12.5 ± 0.9 (ab)	12.2 ± 1 (sb)	12.0 ± 0.8 (b)	11.5 ± 0.7 (b)	4.415 **	0,234	0,024
Sporophyte									
Vaginula length	471.7 ± 60.9 [306.7-625]	476.6 ± 47.2 (bc)	482.5 ± 39.9 (b)	425.1 ± 67.8 (c)	430.7 ± 57.4 (c)	552.1 ± 48.3 (a)	10.256 ***	0,041	-0,279
Seta length	526.1 ± 98 [332-775]	646.8 ± 81.5 (a)	487.5 ± 52.4 (b)	457.3 ± 55.3 (b)	503.5 ± 90.8 (b)	654.6 ± 77.6 (a)	20.355 ***	0,026	-0,204
Capsule length [1]	2 ± 0.2 [1.5-2.6]	2.0 ± 0.1 (b)	2.0 ± 0.2 (b)	2.0 ± 0.2 (b)	1.9 ± 0.1 (b)	2.3 ± 0.2 (a)	7.823 ***	0,031	**-0,350**
Capsule neck length	409.4 ± 86.7 [225-632.5]	536.5 ± 62.7 (a)	406 ± 73.7 (b)	345.5 ± 72.5 (b)	360 ± 55.6 (b)	430.2 ± 49 (b)	13.457 ***	0,189	-0,144
Suboral ring width	123 ± 13.7 [92.5-155]	121.9 ± 7	123.4 ± 14	126.6 ± 16.4	118 ± 15.9	126.9 ± 11.4	0.806	0,172	-0,063
Exotecial band cell length	49.6 ± 7.8 [32.5-68.8]	43.4 ± 4.7 (c)	48.8 ± 6.9 (bc)	53.3 ± 5.8 (ab)	46.4 ± 6.4 (bc)	61.2 ± 4.1 (a)	11.473 ***	-0,156	-0,256
Exotecial band cell width	17.1 ± 2.6 [12.5-23.8]	14.9 ± 1.5 (ab)	17.8 ± 2.5 (a)	17.3 ± 2.2 (ab)	16.5 ± 3.1 (ab)	17.9 ± 1.9 (a)	3.054 *	-0,037	-0,122
Endostome segment length	233.9 ± 33.4 [146.1-355]	212.6 ± 11.1 (b)	232.3 ± 32.5 (b)	230.2 ± 39.1 (b)	234.4 ± 19.6 (ab)	270.9 ± 43.8 (a)	4.103 **	-0,042	-0,218
Spore length	19.3 ± 2.1 [13.8-26]	20.2 ± 1.6	19.3 ± 2.7	18.8 ± 1.2	18.6 ± 2	19.8 ± 0.9	1.083	0,143	-0,061
Spore width	18.4 ± 2.2 [13.1-25.8]	19.6 ± 1.9	18.6 ± 2.8	17.4 ± 1.6	17.5 ± 1.5	18.6 ± 0.8	1.918	0,152	-0,064

Descriptive statistics for quantitative characters are represented for *Orthotrichum acuminatum* (mean ± SD [range]) and for each of the stablished geographical groups (mean ± SD) (all measurements are in µm except those with [1] = cm and [2] = mm). ANOVA F statistic and significance level (* ≤ 0.05, ** ≤ 0.005, ***<0.001) for each variable and five groups is given. Letters (abc) in braquets represent results from post-hoc Tukey Test for the statistically significant variables. PCA component loadings for each original variable are represented. In bold, variables with the highest loadings for each component. Percent of total variance explained for first component (PC1) = 23.46 %, second component (PC2) = 13.86 % and third component (9,53%).

All characters were summarized according to the five geographical regions in the form of beanplot graphs (Kampstra, 2008). Beanplot graphs represent the empirical density shape, mean, and all individual observations for each evaluated group. DFA analysis was performed with SPSS v.22 (IBM Corp.2013) and the rest of the analyses were implemented using the free software R (R Core Team 2013).

DNA extraction and sequencing

DNA was extracted from apices of stems and branches, from dried herbarium specimens. Total DNA was extracted using the DNeasy® Plant Mini Kit for DNA isolation from plant tissue (Qiagen).

After testing the variability of several genomic regions previously used for phylogenetic reconstructions in bryophytes (Stech & Quandt, 2010; Medina R. *et al.*, 2013), we selected the four loci with the greatest variation among those evaluated: two from the plastid genome (*rps4*, *trnL-F*) and two from the nuclear genome (ITS2, *ort-LFY*). The primer pairs used for the *rps4* were *rpsA/trnaS* (Souza-Chies *et al.*, 1997), and for the ITS2 were ITS2*F*/ITS2*R* (Fiedorow *et al.*, 1998). For the *ort-LFY* we used the external primers LFY1428F/ LFY2327R and the internal primers *ort-LFY-R/ort-LFY-R* following Medina R. *et al.* (2013). Primers used for the amplification of *trnL-F* were designed for this study: *trnLc-104* (5'TAAGCAATCCTGAGC3'), and *trnFF-425* (5'CTCTGCTCTACCAACT3').

Double-stranded DNA templates were prepared by PCR, which was performed using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech Inc) in a final reaction volume of 25 μL according to the manufacturer's instructions. The nuclear loci *ort-LFY* was amplified using a nested PCR approach following Medina R. *et al.* (2013). For *rps4* after an initial denaturation step of 5 min at 94°C, 30 cycles were carried out consisting of 30 s denaturation at 95°C, 1 min of annealing at 52°C and a 30 s extension at 68°C, followed by a final extension step of 7 min. For *tnrL-F*, a denaturation step of 5 min at 95°C, followed by 38 cycles of 30 s at 94°C, 1 min at 47°C, 30 s at 72°C and 30 s at 94ºC, and a final extension step of 10 min at 72ºC was employed. For the internal transcribed spacer (ITS2) the PCR program employed consisted of a denaturation step of 1 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min 30 s at 72°C, followed by a final extension step of 5 min. PCR products were purified using Exo/SAP protocol (Thermo Fisher Scientific,

Spain). Samples were incubated with 1 µL of Exo1 enzyme and 4 µL of FastAP following the manufacturer's instructions. Cleaned PCR products were sequenced by Macrogen (www.macrogen.com).

Phylogenetic analyses

Nucleotide sequence contigs were edited and assembled for each DNA region using PhyDE v.0.9971 (Müller *et al.,* 2006). All sequences were aligned manually and trimmed at the ends. Phylogenetic information from indels was coded as an adjacent block with the program SeqState version 1.25 (Müller, 2005) using the simple indel coding method (Simmons & Ochoterena, 2000). All phylogenetic analyses were performed with and without codified indels.

Phylogenetic analyses were performed using maximum parsimony (MP) and Bayesian inference (BI). Maximum parsimony analyses were performed using the program TNT 1.0 (Goloboff *et al.,* 2003). Swapping algorithm selected was tree bisection reconnection (TBR). All characters were equally weighted. Clade support was assessed via non-parametric bootstrapping (BS) using the default settings in TNT, except for the number of replicates, which was set to 1000. For all generated MP trees the consistency index (CI) and retention index (RI), as well as tree length were retained.

BI phylogenetic analyses were carried out using MrBayes v.3.2.1 (Ronquist *et al.*, 2012). Analyses were done on a partitioned matrix: one partition for each locus plus a fifth partition for the coded indel block. The best-fitting substitution models for each matrix locus (Table 3.1.2) were inferred under the Bayesian Information Criterion (BIC) in jModelTest v.2.1.3 (Darriba *et al.,* 2012). For the indel block the F81 model was applied following Ronquist *et al.* (2005). The Markov Chain Monte Carlo (MCMC) simulation was executed with two runs and four chains, until the standard deviation of split frequencies was below 0.01 (5,000,000 generations for the combined plastid and nuclear genome matrix). Trees and parameters were sampled every 1000th generation. Posterior probabilities (PP) were estimated from the 50% majority-rule consensus trees after a burn-in of 25% of the starting trees, and plotted using FigTree v.1.4.2 (Rambout, 2012).

All the BI and MP phylogenetic analyses were first conducted on the plastid (*rps4* and *trnL-F*) and nuclear (ITS2 and *ort-LFY*) data sets separately. To test for incongruence

between plastid and nuclear partitions, a visual inspection of the independent phylogenetic trees was performed. High levels of support (BS, PP) are used as assessment of robustness (Huson & Bryant, 2006). Branches supported with PP ≥ 0.95 and BS ≥ 85, were congruent in all separate analyses, and therefore final analyses were run on a concatenated matrix, where missing sequences were omitted.

Results

Morphological analyses

The overall variation obtained for the qualitative morphological characters across the 74 samples matches the one described for *Orthotrichum acuminatum* in the Iberian Peninsula by Lara & Garilleti (2014). The qualitative diagnostic characters for the species are constant within and among the three regions, including those related to the hygrocastique spore release (capsule shape, prominence of capsule ribs, and endostome segments; see Fig. 3.1.3, Table 3.1.S1). Specifically, 51 out of the 60 qualitative traits are constant within and among regions, whereas the remaining nine characters display a slight variation but with no geographical pattern or taxonomic relevance (Table 3.1.S1). Two characters (annulus and exostome teeth cell number) are slightly different in California. Three other traits (leaf margin, capsule ribs when wet, and calyptra hairiness) show more variation in the Mediterranean. Two other traits (rhizoids position on stems and length of exothecial bands) differ somewhat in Ethiopia. The remaining two variable characters are different among the three regions (vaginula hairs and polysety); both traits vary in parallel, since the presence of short hairs in the vaginula and more than one sporophyte per perichaetium is common in California, occasional in the Mediterranean, and exceptional in Ethiopia. In some characters the variation found within a region or even among individuals of a population is very broad. This is the case of the variation obtained for both upper and perichaetial leaf apex shapes, but the results do not reveal any differentiation among regions.

Quantitative traits display a higher level of intra- and inter-regional morphological variation than the one detected among the qualitative ones. Twenty out of the 27 quantitative characters show statistical differences among the five geographical sub-regions (Table 3.1.1). Descriptive statistics show slight geographical tendencies when variables are considered independently (Table 3.1.1, Figs. 3.1.4, 3.1.5, 3.1.S1). Californian specimens

have significantly higher mean values for 10 gametophyte traits (six from leaves and four from leaf cell sizes), while Ethiopian samples show smaller values for seven of these traits (Table 3.1.1, Figs. 3.1.4, 3.1.S1). As for the sporophyte characters, seta is usually longer in California and Ethiopia than in the Mediterranean samples, vaginula and capsule tend to be longer in Ethiopia, and capsule neck is longer in California (Table 3.1.1, Fig. 3.1.5). The three Mediterranean sub-regions display intermediate mean values between the ones described in California and Ethiopia for 12 of the 27 variables. However, the Mediterranean Basin shows the highest range of variation, enclosing the values found in California and Ethiopia (Table 3.1.1, Figs. 3.1.4, 3.1.5, 3.1.S1).

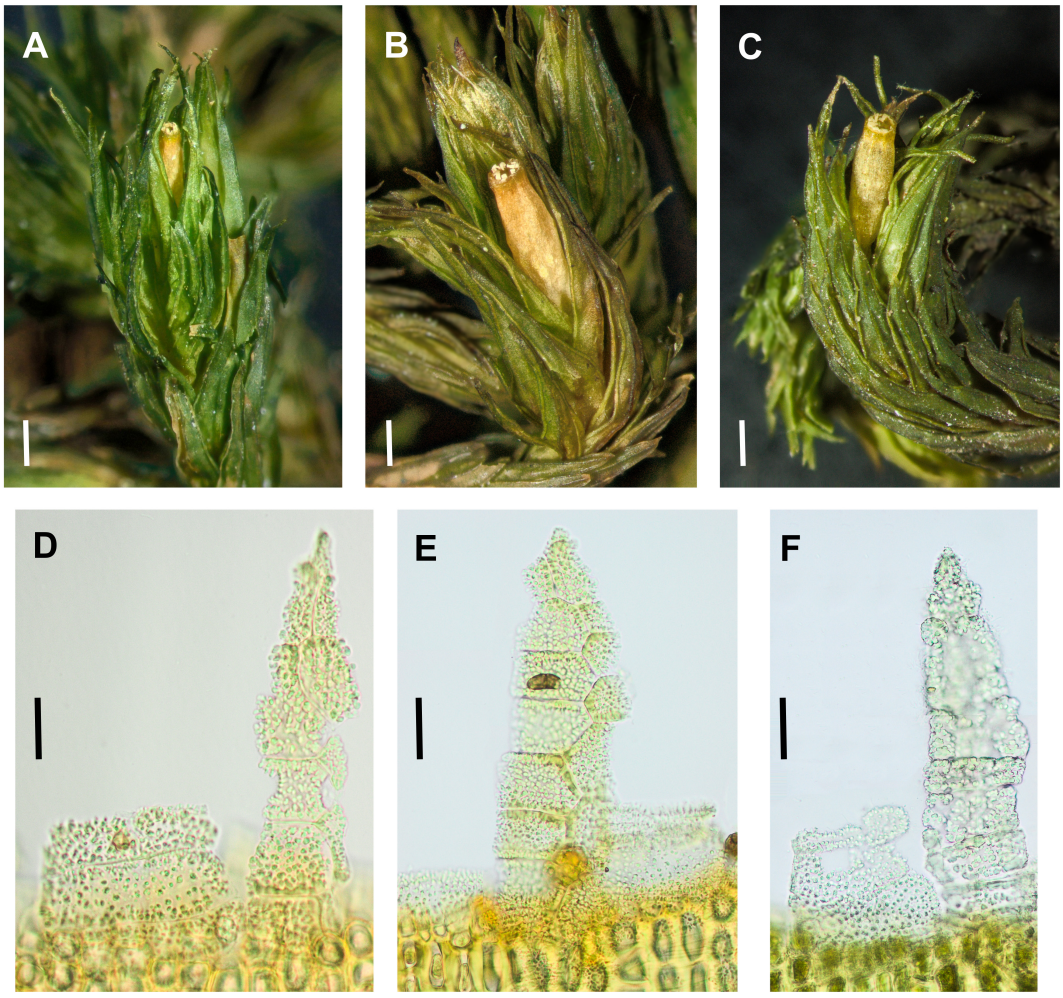


Figure 3.1.3. Images of *Orthotrichum acuminatum* from the three main studied regions. A, D: California (MAUAM-Brio 3318, 3319); B, E: Mediterranean Basin (Spain: MAUAM-Brio 3155); C, F: Ethiopia (MAUAM-Brio 3308). A-C: habit, detail of apical part of shoot with mature open capsules. D-F: portion of the peristome, showing a rudimentary exostome tooth and a complete endostome segment. Scale bars: A-C= 500 µm, D, F = 25 µm, E = 30 µm.

In the PCA results, the two first principal components (PCs) accounted for 37.32% of the variance (Fig. 3.1.6). A vague geographical structure is shown by the PCA since the five geographical sub-regions overlap to some degree. Samples from the three Mediterranean sub-regions appear scattered across the ordination intermingling with each other. All the Californian samples are fairly grouped but overlapping with mainly Western Mediterranean samples. Similarly, the Ethiopian samples are grouped together but overlapping with some other Western Mediterranean samples. The most important variables in PC1 (loading values ≥ 0.3, Table 3.1.1) are those related to lower and upper leaf sizes. These variables show larger values for all the Californian samples and part of the Mediterranean. The most important variables according to PC2 are the acumen length of the upper leaves, the perichaetial leaf length and the capsule length. All the Ethiopian samples have high values for these three variables (Table 3.1.1, Fig. 3.1.6). The DFA analysis for the three main regions shows significant differences among groups (Wilks' $\lambda = 0.040$, 0.221 and $P < 0.001$, $P < 0.001$). In this case, 89.2% of cross-validated grouped samples are correctly classified (Table 3.1.S2). For the five groups analysis, the DFA reveals significant differences for three of the four discriminant functions (Wilks' $\lambda = 0.010$, 0.065, 0.305 and 0.571; $P < 0.001$, $P < 0.001$, $P = 0.048$ and 0.127, respectively). Cross-validation results indicate that 56.8% of the grouped cases are correctly classified (Table 3.1.S3). This low correctly assigned classification is due to errors in the classification of samples from the three Mediterranean sub-regions (Western = 53.1%, Central = 30% and Eastern = 50%), whereas Californian and Ethiopian samples are better classified (80% and 87.5% respectively): two specimens from California and one from Ethiopia are classified as Western Mediterranean, and three specimens from Western Mediterranean are classified as Californian (two specimens, 9%), and Ethiopian (one specimen, 12.5%). Finally, one specimen from Eastern Mediterranean is classified as Ethiopian (3.1%). Significant differences among the five geographical groups are also assessed with MANOVA (Wilks' $\lambda = 0.010$, $F = 3.562$, $P < 0.001$). However, post-hoc test for unequal sample size does not reveal any congruence in the groups detected among variables, since for all variables California, Ethiopia or both appear clustered with the Mediterranean except for the variable seta length (Table 3.1.1, Figs. 3.1.4, 3.1.5, 3.1.S1).

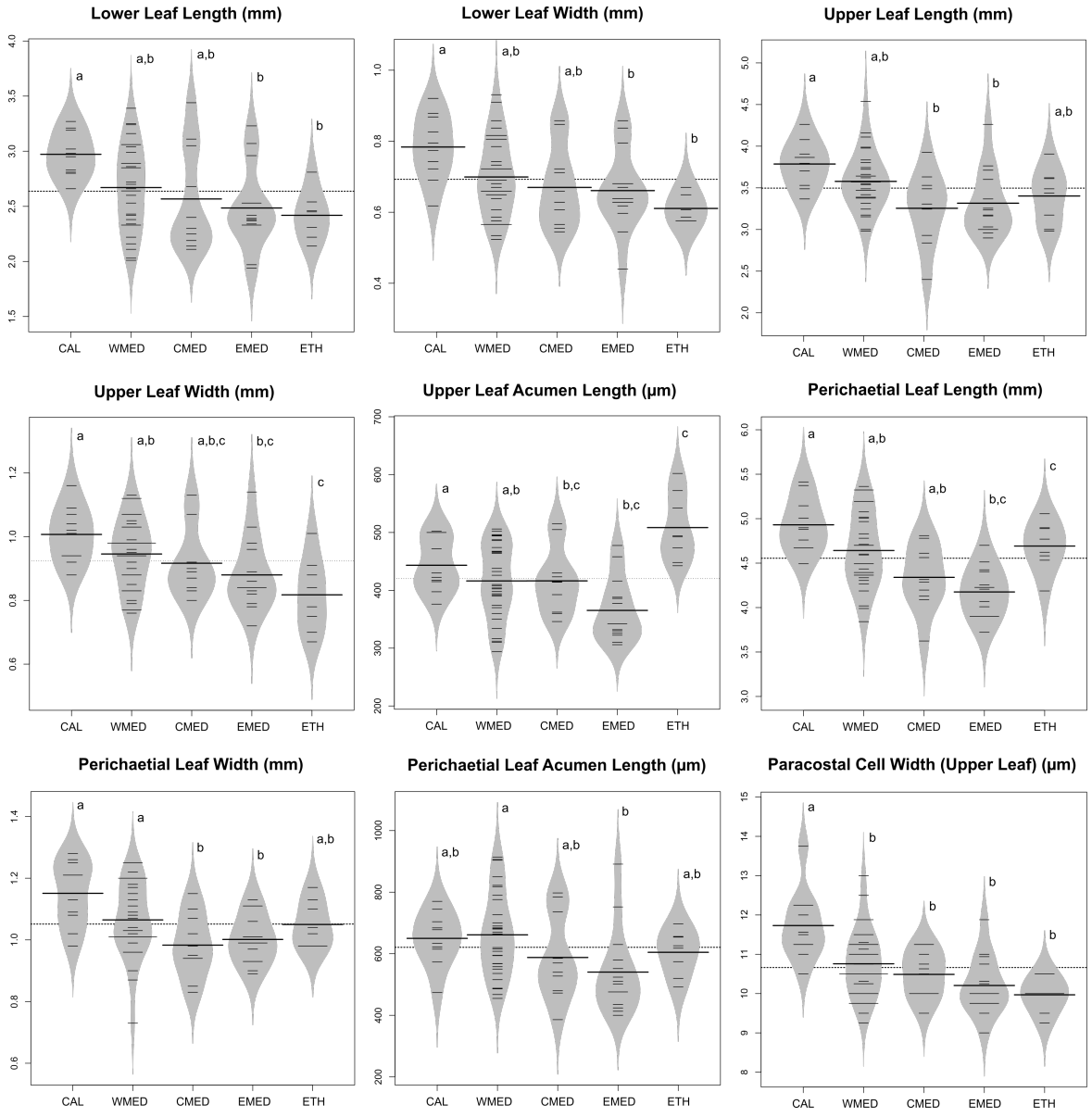


Figure 3.1.4. Beanplots of the most informative gametophyte quantitative variables of *Orthotrichum acuminatum* for each geographical region. Individual observations are represented by small horizontal lines (in case of multiple observations with the same values, corresponding number of lines were merged), mean per group is shown by bold long line and mean for all data by a dotted line. Estimated density of the data distribution is displayed by the density shape in grey (for details see Kampstra, 2008). Letters (a,b,c) represent groups with significant differences revealed by post hoc analysis for unequal sample size. CAL = California, WMED = Western Mediterranean, CMED = Central Mediterranean, EMED = Eastern Mediterranean and ETH = Ethiopia.

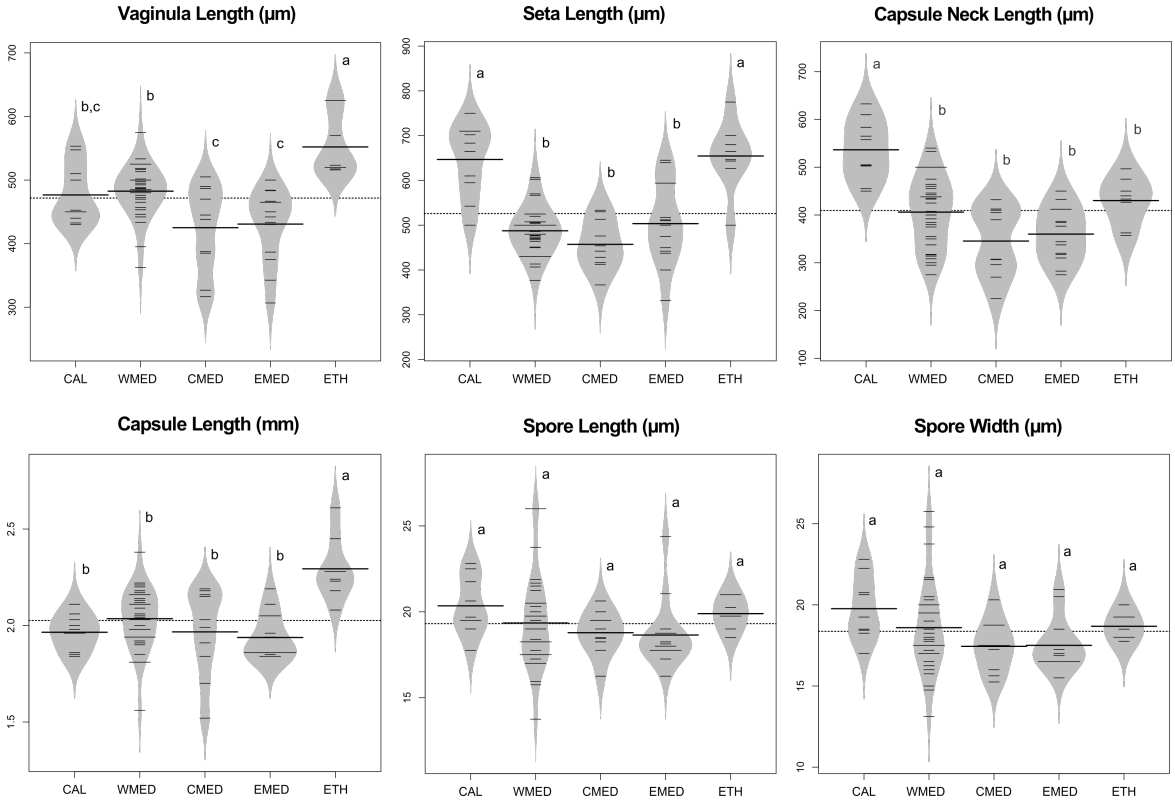


Figure 3.1.5. Beanplots of the most informative sporophyte quantitative variables of *Orthotrichum acuminatum* for each geographical region (see Figure 3.1.4).

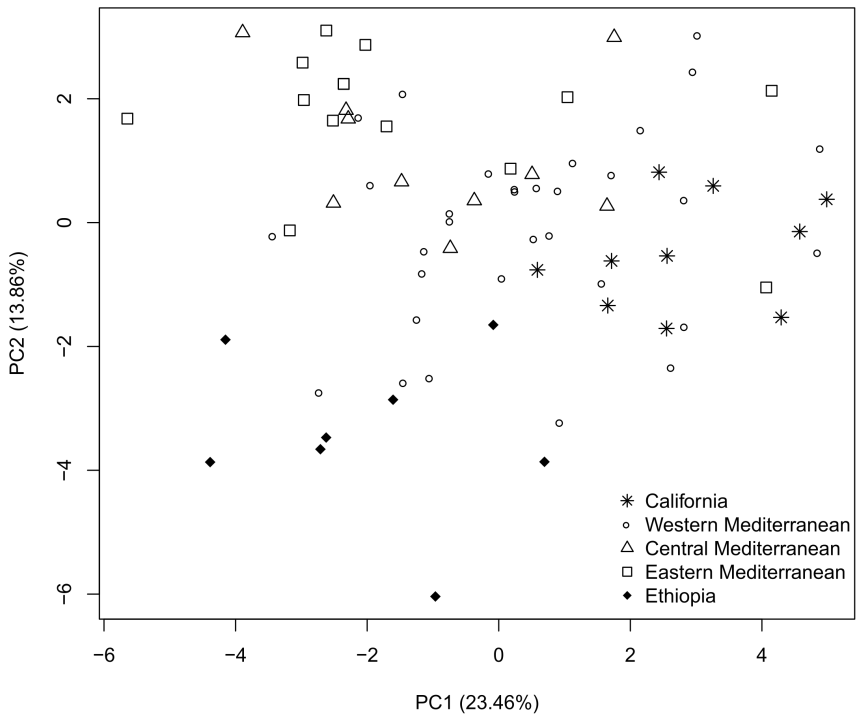


Figure 3.1.6. Results of the principal component analysis (PCA) for *Orthotrichum acuminatum* specimens. The percentage of variance explained by each component is given between brackets. Symbols represent the five established geographic areas as in Figure 3.1.1.

Phylogenetic analyses

The combined alignment of the four plastid and nuclear loci plus the indel block is 3802 characters long (*rps4* 668 bp; *trnL-F* 325 bp; ITS2 551 bp; *ortLFY* 1902 bp; indel block 356 binary characters). The total number of variable and parsimony-informative positions in the combined matrix including indels is 617 and 311, respectively (Table 3.1.2). Within *Orthotrichum acuminatum*, three (without indels) and seven (with indels) variable sites are displayed, only one of them parsimony-informative.

Both MP and BI analyses of the combined matrix, with and without codified indels, are congruent. As the analyses conducted with the indels codified show higher resolution and support values, hereafter we will refer to them. Phylogenetic analyses resolve *Orthotrichum acuminatum* as a monophyletic group (PP=1.0, BS=100), which includes all samples from the five geographic areas studied (Fig. 3.1.7). A large basal polytomy is recovered within *O. acuminatum*, including samples from Ethiopia, central and Eastern Mediterranean and the one from the Canary Islands. Within this polytomy also appears a well-supported monophyletic subclade (PP=0.99, BS=61) that includes samples from Western Mediterranean and California. This polytomy is soft due to the lack of informative characters. Indeed, the single informative character retrieved in *O. acuminatum* supports the Californian-Western Mediterranean subclade (Table 3.1.2, Fig. 3.1.7). Considering the outgroup sampling, *Orthotrichum affine* Brid. appears to be the sister group of *O. acuminatum* (PP = 0.99, BS=59).

Table 3.1.2. Characteristic of the four DNA regions sequenced for phylogenetic analyses. Information between brackets corresponds to results including codified indels.

	Nuclear data		**Plastid data**		
	ITS2	***ort*LFY**	***rps*4**	***trn*L-F**	**Combined**
Sequences	30	29	30	29	28
Aligned length (with indels)	551 (653)	1902 (2132)	668 (682)	325 (340)	3446 (3802)
Gap sites	102	230	14	15	356
Total matrix					
Variable sites	52 (66)	84 (186)	47 (55)	37 (52)	392 (614)
Potentially informative sites	19 (23)	28 (53)	13 (15)	15 (20)	202 (311)
Orthotrichum acuminatum					
Variable sites	0 (0)	1 (5)	2 (2)	0 (0)	3 (7)
Potentially informative sites	0 (0)	1 (1)	1 (1)	0 (0)	1 (1)
Substitution model (BIC)	HKY+G	HKY+G	HKY+I	HKY+G	

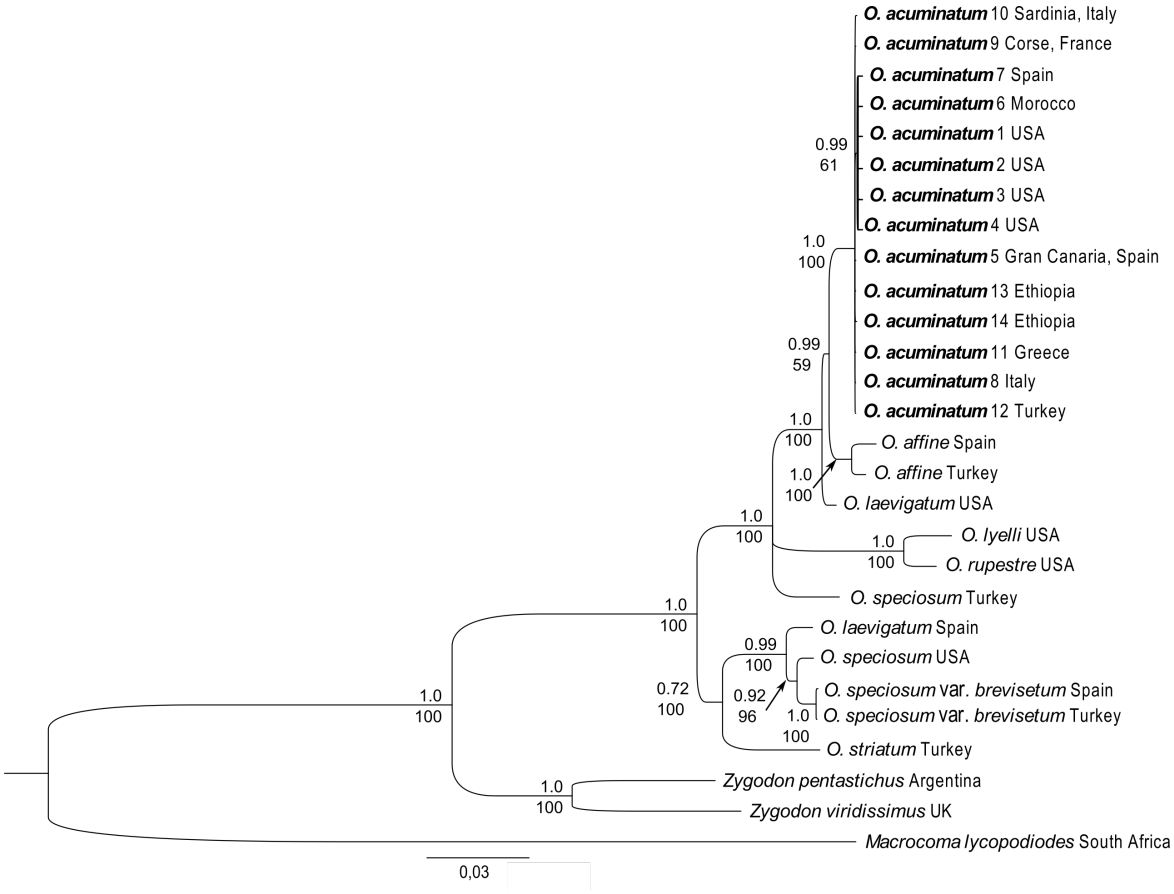


Figure 3.1.7. Majority-rule consensus tree of the Bayesian analysis obtained from the concatenated DNA matrix including two plastid (*rps*4, *trn*L-F) and two nuclear (ITS2, *ort*-LFY) loci, considering indels. Numbers above branches indicate Bayesian posterior probabilities. Bootstrap supports obtained in the Maximum Parsimony analysis are given below branches. Individuals of the same species are named as in Appendix (see also Fig. 3.3.1 for geographic location of *Orthotrichum acuminatum* individuals).

Discussion

Recognition and validation of cryptic species is a major challenge to modern taxonomy with deep implications in biogeography. The study of cryptic species in bryophytes usually reveals morphological uniformity with an underlying complex phylogenetic and genetic structure (Feldberg *et al.*, 2007; Vanderpoorten *et al.*, 2008; Laenen *et al.*, 2011). In the case of broadly distributed species, different studies have reported that what appears to be a morphologically uniform widespread species, actually consists of several cryptic or sibling species (e.g. Hutsemékers *et al.*, 2012). Within *Orthotrichum*, some recent studies have revealed more subtle (Medina R. *et al.*, 2012) or clearer (Medina R. *et al.*, 2013) morphological differentiation together with a congruent genetic structure, leading to the split of species previously considered disjunct into several taxa. However, our results show that

Orthotrichum acuminatum populations from the Mediterranean, Western North America and Ethiopia are morphologically homogeneous considering qualitative characters (Table 3.1.1, Fig. 3.1.3). The only noticeable qualitative differences are the common presence of some vaginula hairs and polysety in Californian specimens, which are only occasional in the Mediterranean ones, and exceptional in the Ethiopian ones. Nevertheless, *Orthotrichum* species with glabrous vaginula but hairy calyptra, occasionally show sparse hairs in the vaginula (Plášek & Sawicki, 2010), and this variation cannot be considered enough to recognize any taxonomic entity. Polysety rarely has been used as a taxonomical character within the genus because of its inconstancy in the species where it appears (Lewinsky 1993; Lewinsky-Haapasaari & Hedenäs 1998).

Quantitative morphological analyses reveal slight tendencies of differentiation among populations. DFA analyses indicate significant differences among the five established geographic sub-regions. However, the degree of variation detected in California and Ethiopia lies within the range of variation described in the Mediterranean Basin itself, and the mentioned tendencies do not show a constant pattern of deviation among geographic areas to support any geographic differentiation. These morphological results agree with the phylogenetic reconstructions, since no geographic structure has been established among the Mediterranean, Californian and Ethiopian populations (Fig. 3.1.7). Additionally, we have found very low levels of genetic variation in all the studied DNA regions among the disjunct populations (Table 3.1.2). This pattern of morphological uniformity, coupled with little or no molecular differentiation among populations on different continents, has been repeatedly reported for bryophytes (Shaw, 1993, 2001; Shaw *et al.*, 2003; Werner *et al.*, 2003; Stech & Dohrmann, 2004; Stenøien *et al.*, 2011). Therefore, we consider that the recently found populations from California and Ethiopia correspond to *O. acuminatum*, since we have not found enough morphological or molecular evidence that could support taxonomic differentiation. Consequently, these findings document a new case of intercontinental disjunction among bryophytes.

This new geographic pattern discovered for *Orthotrichum acuminatum*, a Western Palearctic–Western Nearctic–Eastern Africa disjunction, seems to have no parallel within bryophytes, although some species with a somewhat wider distribution are known to be present in these three areas of the world (e.g. Hedenäs, 2008). A different situation arises when considering each of the intercontinental disjunctions into which the range of *O.*

acuminatum could be broken down separately. On the one hand, the Western Palearctic–Western Nearctic disjunction (also called Western North America-Western Europe, Madrean–Tethyan, or Mediterranean–Californian disjunction) is well known among bryophytes. After Schofield (1988), this disjunction accounts for approximately 7% of the European and 6% of the North American mosses, and approximately 5% of the European and 4% of the North American liverworts. On the other hand, several bryophytes widespread in temperate areas of the Northern Hemisphere are known to be present in Eastern Africa (e.g. Bizot *et al.*, 1978; Hedenäs 2008). However, no biogeographical studies refer to a strict Mediterranean-Eastern Africa distribution in bryophytes.

The occurrence of *Orthotrichum acuminatum* in Ethiopia raises the question of whether other non-widespread bryophyte species are present primarily in the Mediterranean Basin, as well as elsewhere in Eastern Africa. A comparison of the checklist of the mosses of sub-Saharan Africa (O'Shea, 2006) with the data from the European species with higher Mediterranean affinities used by Medina N.G. *et al.* (2011), showed that 17 out of 117 (14.5%) of the Mediterranean species also occur in Eastern Africa. This suggests that Eastern Africa is a very important area for this type of species; even though it lacks a Mediterranean climate, it holds more species typical of the Mediterranean Basin than other Mediterranean climate areas of the world, except for California (Medina N.G. *et al.*, 2011). Furthermore, eight of the 17 Mediterranean-Eastern African species also appear in California, and four of them seem to be restricted to these three areas, namely *Entosthodon convexus* (Spruce) Brugués, *Epipterygium tozeri* (Grev.) Lindb., *Syntrichia laevipila* Brid., and *Timmiella anomala* (Bruch & Schimp.) Limpr. As a result, the global distribution pattern reported here for *O. acuminatum*, although infrequent, is not unique.

Turning to the Western Palearctic–Western Nearctic disjunction, it is well known in vascular plants, with a large number of studies supporting the hypotheses of LDD and different migration pathways to explain this pattern (Wen & Ickert-Bond, 2009; Kadereit & Baldwin, 2012; Vargas *et al.*, 2014). In bryophytes, some authors consider intercontinental disjunctions like this to be the result of ancient vicariance combined with morphological stasis and slow evolutionary rates (see, for instance, Frey *et al.*, 1999). In contrast, other studies suggest a more recent origin through LDD as the most likely mechanism underlying biogeographical scenario for the Western Palearctic–Western Nearctic disjunction (Shaw *et al.*, 2003; Werner *et al.*, 2003; Huttunen *et al.*, 2008). Furthermore, LDD has been proposed

for several bryophyte species showing other wide intercontinental disjunct patterns, such as Bipolar (Piñeiro *et al.,* 2012), Trans-Antarctic (McDaniel & Shaw, 2003), Amphi-Pacific (Shaw *et al.,* 2013) or Amphi-Atlantic (Stenøien *et al.,* 2011). Morphological homogeneity and genetic patterns herein described for *Orthotrichum acuminatum* may also suggest LDD as the most plausible explanation for the origin of the Californian populations, since the little sequence variation detected is hardly expected within the c.a. 25 Myr context of the separation of Europe and North America (Shaw *et al.,* 2002). However, migration through different pathways (Wen & Ickert-Bond, 2009) cannot be discarded a priori, although it seems unlikely given the ecological preferences of *O. acuminatum*.

As for the Western Palearctic and Eastern Africa disjunction, similarities and connections between the two regions are common in vascular plants at a generic level (e.g. Rand Flora elements: Andrus *et al.,* 2004; Sanmartín *et al.,* 2010), and more rarely at a species level (Désamoré *et al.*, 2011). Some authors propose this disjunction as a vicariance resulting from the fragmentation of the dominating Tertiary flora due to climatic changes that took place during the Miocene and Pleistocene (Désamoré *et al.,* 2011; Pokorny *et al.,* 2015). At the same time, other studies conclude that species migrated to or from Eastern Africa through long distance cross-continental dispersion processes (Assefa *et al.,* 2007; Pelser *et al.,* 2012), in some cases by stepping-stone via intermediate mountain systems, which provided suitable habitats during favorable climate periods. For bryophytes, Hedenäs (2008) suggests that the genetic variation found in African populations of *Antitrichia curtipendula* results from the isolation of these populations due to the climatic fluctuations during the Pleistocene. However, no genetic variation has been found in *Orthotrichum acuminatum* between the Mediterranean and Ethiopian populations in the studied loci. This, together with the lack of considerable morphological variation among populations, may reflect that in *O. acuminatum* this disjunction is probably due to recent LDD events, as also suggested above for the Western Palearctic–Western Nearctic disjunction.

Interestingly, *Orthotrichum acuminatum* has hygrocastique spore release, a mechanism that requires high environmental humidity for spores discharge – during or just after rain–. This strategy probably enables rapid germination, but also may limit the efficiency of spores for LDD (Mueller & Neumann, 1988). A rapid germination is supposed to be advantageous either when the spores are intolerant to water stress periods, or when favorable environmental conditions are ephemeral. This has been interpreted as a safe-site strategy

(Medina N.G. & Estébanez, 2014) intended to favour moss establishment. Epiphytic species thriving in Mediterranean environments, such as *O. acuminatum* (Lara *et al.*, 1999), are exposed to strong seasonal aridity and to unpredictable inter-annual rain fluctuations. *Orthotrichum acuminatum* may thus take advantage of its hygrocastique mechanism to survive in this type of conditions, which may well explain its great success in population expansion and establishment throughout the Mediterranean Basin. On the other hand, small and resistant spores able to reach, at least occasionally, the high layers of the atmosphere that are needed for LDD to occur (Van Zanten, 1978; van Zanten & Pócs, 1981). *Orthotrichum acuminatum* shows a wide range of spore sizes (14-26 µm in diameter, see Table 3.1.1), with a considerable fraction of spores small enough (~80% < 20 µm in diameter) to be easily carried by wind among continents (Wilkinson *et al.*, 2012). Moreover, Medina N.G. & Estébanez (2014) reported between 10% and 20% of bicellular spores in this species. Bicellular spores have originated by precocious endosporic germination that involves, before spore release, an initial sporeling development inside the spore cell wall. These spores tend to be somewhat larger and, since they are in a more advanced stage than unicellular ones, they are presumably ready to complete the protonemal development immediately after release (Garilleti *et al.*, 2012). Consequently, it seems likely that *O. acuminatum* performs a dual dispersal strategy: 1) by means of bicellular spores, whose size is usually considered too big for LDD (but see Sundberg, 2013), it ensures a rapid and nearby gametophyte development when environmental conditions are favorable; and 2) through the most frequent small spores (< 20 µm) it is capable of being dispersed over long distances more often.

The Mediterranean Basin could be considered the main source of dispersion for *Orthotrichum acuminatum* since it is a large area where the species is widespread and occupies a large variety of habitats. Although speculative, this idea is suggested by our results, since the studied specimens from the Mediterranean Basin show the greatest morphological variation, enclosing that one found in California and Ethiopia (Table 3.1.1, Figs. 3.1.4, 3.1.5, 3.1.S1). The existence of several *O. acuminatum* populations in Southern California along the Peninsular Range (San Jacinto and San Bernardino Mountains) highly contrasts with the scarce, nearby localities found in the Simien Mountains in Ethiopia. Our research team has largely surveyed neighbouring suitable areas in both regions (northern California, Baja California, other Ethiopian mountain zones, Kenya and Tanzania

mountains) without finding further populations of this moss. In both cases, the extend of the *O. acuminatum* distribution is considerably limited, although in California the species seems to be more firmly established because of the higher number of populations and diversity of occupied habitats. The morphological analyses have revealed a relative homogeneity within both California and Ethiopia, and a slight differentiation of the populations from these areas with regard to those from the Mediterranean Basin. The morphological evenness could be related to a limited phenotypic variation due to a lesser environmental variation in the extra-Mediterranean areas, which is especially evident in Ethiopia, where a unique ecosystem type (in fact, a single large woodland) has been colonized. In turn, the little but actual morphological differentiation found among disjunct populations could reflect incipient speciation. Finally, these morphological results are also consistent with a recent colonization of these extra-Mediterranean areas by means of a limited number of propagules. Nevertheless, the scarce sequence variation of the DNA regions here employed did not allow performing genetic diversity analyses to confirm or reject any of these hypotheses.

The origin of the North American populations might be the Western Mediterranean, given the phylogenetic placement of the Californian specimens within the subclade including the samples from Morocco and the Iberian Peninsula (Fig. 3.1.7). This relationship is also supported by the morphological similarity of the samples from these two regions in some of the variable quantitative characters (Fig. 3.1.6, Tables 3.1.1, 3.1.S3). For the Ethiopian samples, our molecular data do not allow to conclude about their possible origin, since they appeared intermingled with the rest of the samples studied in the phylogenetic tree (Fig. 3.1.7). However, according to their morphology, they are also more similar to the Western Mediterranean specimens than to any other (Fig. 3.1.6, Table 3.1.S3).

On the basis of the results above discussed, we discard our working hypothesis of the existence of a complex of cryptic species underlying the newly discovered intercontinental disjunct populations of *Orthotrichum acuminatum*. Instead, *O. acuminatum* should be considered as a new documented case of intercontinental disjunction within bryophytes, with populations in three different continental and biogeographic areas: the Mediterranean Basin (Western Palearctic), Western North America (Western Nearctic) and Eastern Africa (Paleotropical). However, although we suggest LDD as the origin of this distribution pattern, new approaches are needed –such as phylogeographic, population genetic studies and divergence age estimates– to confirm or reject this hypothesis, and provide a timeframe for

the disjunctions in order to reach robust biogeographic conclusions on the origin and expansion of *O. acuminatum.* Furthermore, ecophysiological experiments should be performed to test spore resistance and germination rates under different extreme conditions, as well as to assess the relationship between the hygrocastique spore release traits and *O. acuminatum* capabilities for long distance dispersal.

Acknowledgements. We thank Jim Shevock for his help during the field work across the southern California Mountains, which made possible the first findings of *Orthotrichum acuminatum* in this territory. In Ethiopia, Binyam Getachew was much more than a driver for us, and his invaluable guidance helped to find the best sites to prospect. To him we address our warm thanks as well. We finally wish to address our thanks to the anonymous referees whose critical revisions helped to improve the quality of the manuscript, and to B. Garrido for revising the English text. Boards of the California Department of Parks and Recreation and the Simien Mountains National Park are gratefully acknowledged for collecting permissions. This research was supported by the Spanish Ministries of Education and Science (CGL2007-61389/BOS), Science and Innovation (CGL2011-28857/BOS), and Economy and Competitiveness (CGL2013-43246-P). B.V. benefited from the support of the Ministry of Economy and Competitiveness through grant BES-2012-051976 of the Formación de Personal Investigador (FPI) programme.

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Appendix

Selected specimens

All specimens listed below have been used for morphological analyses. Those included in molecular analyses are followed by GenBank accession numbers [ITS2 / *ort*-LFY / *rps*4 / *trn*L-F]. Numbers in bold preceding molecular information correspond to the specimens of *O. acuminatum* used in illustrations of Figs. 3.1.1 and 3.1.7.

***Orthotrichum acuminatum*. Cyprus:** Östliches, Tróodos-Gebirge, Mt. Kionia, 23 May 2002, *Schäfer-Verwimp 22791* (VAL-Briof s / n); Westliches Tróodos Gebirge, Umgebung Stavros Forestry Station, Kiefernwald, 20 Feb 2009, *Frahm 2009745*, MAUAM-Brio 3161; **Ethiopia:** Simien Mountains. Ambaras, Jinbar River, 20 Nov 2013, *Lara & Vigalondo*, MAUAM-Brio 3309 [**15:** *KT862267 / KT862354 / KT862297 / KT862326*]; Ambaras, Jinbar River, lateral valley, 20 Nov 2013, *Lara, Mazimpaka & Vigalondo*, MAUAM-Brio 3308 [**14:** *KT862266 / - / KT862296 / KT862325*]; *Erica* forest below Geech Camp, 18 Nov 2013, *Lara, Mazimpaka & Vigalondo*, MAUAM-Brio 3307, 3310, 3311, 3312 [**13:** *KT862265 / KT862353 / KT862295 / KT862324*]; **France:** Corse. Golo River Valley, between Ponte Castirla and Francardo, 27 Sep 2004, *Lara*, MAUAM-Brio 3162; *Juniperus* forest of Asco, Valle Pinara, 23 Sep 2004, *Lara & San Miguel*, MAUAM-Brio 3294; Monte Cintu, between Asco and Haut Asco, bank of the river Asco, 23 Sep 2004, *Lara*, MAUAM-Brio 3164 [**9:** *KT862262 / KT862350 / KT862292 / KT862321*]; **Greece:** Creta, Lasithi, Oropedio lasithiou, Psychro, 14 Aug 2005, *Medina*, MAUAM-Brio 2395; Makedonia, Pieriá, Litóhoro, Mt. Olympos, 5 Aug 1999, *Lara & Mazimpaka*, MAUAM-Brio 3299; Pelopónissos, Ahaia, Oros Chelmos, Vouraikos valley, 21 Mar 1999, *Cano, Muñoz, Ros & Sabvlojevic*, MAUAM-Brio 3130; Stereá Elláda, Fokída, Brallos, to Iti, 27 Jul 1999, *Lara, Mazimpaka & Cano*, MAUAM-Brio 2072; Thráki, Alexandropolis, between Essími and Leptokariá, 4 Aug 1999, *Lara, Mazimpaka & Cano*, MAUAM-Brio 2079 [**11:** *KT862268 / KT862355 / KT862298 / KT862327*]; **Italy:** Alpes, Piemonte. Verbania, Lake Magiore, between Piancassone and Viggiona, 18 Jul 2013, *Lara*, MAUAM-Brio 3305, 3306 [**8:** *KT862269 / KT862356 / KT862299 / KT862328*]; Sardinia. Foresta di Monte, Arcosen, 17 Mar 2008, *Lara*, MAUAM-Brio 3167 [**10:** *KT862261 / KT862349 / KT862291 / KT862320*]; Sicily. Madonie, Piano Zucchi, 27 Jul 1998, *Lara, Garilleti & Mazimpaka*, MAUAM-Brio 3132, 3134; **Morocco:** Alto Atlas, Jbel Touchka, 18 Jun 2000, *Draper, Lara & Mazimpaka*, MAUAM-Brio 3214; Antiatlas, Jbel Lekst, 20 Jun 2006, *Draper, Lara & Mazimpaka*, MAUAM-Brio 3184; Bab Taza, base of J. Bouhalla, 29 Mar 1994, *Garilleti, Albertos, Lara & Vergara*, MAUAM-Brio 3224; Bab Taza, Jbel Bouhalla, 30 Mar 1994, *Garilleti, Albertos, Lara & Vergara*, MAUAM-Brio 3226; Bab Taza, way to J. Bouhalla, 29 Mar 1994, *Garilleti, Albertos, Lara &*

Vergara, MAUAM-Brio 3220; Bab Taza, way up to J. Bouhalla, 30 Mar 1994, *Garilleti, Albertos, Lara & Vergara*, MAUAM-Brio 3223; Ketama, Jbel Bou Bessoui, 16 Mar 1997, *Cano, Gallego, Garilleti, Lara & Ros*, MAUAM-Brio 3230; Medio Atlas, Djebel Bou Ibalne, between Tizi-n' Tiskine and Taffert, 14 Jun 1998, *Cano, Muñoz & Ros*, MAUAM-Brio 3200 [**6:** *KT862275 / KT862357 / KT862300 / KT862329*]; Medio Atlas, way up to Azourki, close to Ouzoud, 24 Jun 2004, *Draper & Medina*, MAUAM-Brio 3205; Taza, way up to Jbel Tazzeka from Bab Bou Idir, 21 Jun 1997, *Albertos, Cano, Coy, Mazimpaka & Ros*, MAUAM-Brio 3244; Rif Mountains, road from Bab Berred to Issaguen, 27 Mar 2013, *Lara*, MAUAM-Brio 4412; **Portugal:** Tras-os-Montes e Alto Douro, Serra da Nogueira, Lançao, 23 Dec 2000, *Garcia, Garilleti, Lara & Mazimpaka*, MAUAM-Brio 2892; **Spain:** Albacete. Yeste, proximities of Tus, 1 Nov 1993, *Lara & Garilleti*, MAUAM-Brio 3159; Almería. Sierra Alhamilla, 8 Feb 1996, *Albertos, Garilleti, Lara & Mazimpaka*, MAUAM-Brio 3298; Sierra Alhamilla, 10 Nov 2005, *Medina*, MAUAM-Brio 3302; Ávila. El Tiemblo, El Castañar, 11 Sep 2012, *Lara*, MAUAM-Brio 3272 [**7:** *KT862263 / KT862351 / KT862293 / KT862322*]; Barcelona. Prepirineo, Collsacabra, Rupit, 28 Sep 2013, *Calleja*, MAUAM-Brio 3301; Cáceres. Cuacos de Yuste, Centro de Interpretación, 12 Apr 2012, *Lara*, MAUAM-Brio 3155; Canary Islands. Gran Canaria, Caldera de los Marteles, 24 Jun 2012, *Vigalondo & Calleja*, MAUAM-Brio 3142; Gran Canaria, way to Roque Nublo, 24 Jun 2012, *Vigalondo & Calleja*, MAUAM-Brio 3144 [**5:** *KT862264 / KT862352 / KT862294 / KT862323*]; La Gomera, Fortaleza de Chipude, 5 Feb 2005, *Lara*, MAUAM-Brio 3331; Ciudad Real. Viso del Marqués, 14 Jun 2006, *Estébanez, N.G. Medina & R. Medina*, MAUAM-Brio 4065; Guadalajara. Tamajón, 13 Feb 2004, *Medina*, MAUAM-Brio 3295; Huesca. Escalona, entrance to Añisclo, 12 Jul 1998, *Garilleti, Lara, Albertos & Cano*, MAUAM-Brio 1626; Jaén. Martos, Sierra de Víboras, 6 Apr 2012, *F. Lara & J. Lara*, MAUAM-Brio 3152; Lleida. El Congost de Montrebei, 25 Jan 2013, *Vigalondo*, MAUAM-Brio 3273; San Esteve de la Sarga, 18 Jun 2005, *Draper, Estébanez & Medina*, MAUAM-Brio 3140; Sierra del Cadí, Barranco Ortedó, 17 Jul 1998, *Albertos, Cano, Garilleti & Lara*, MAUAM-Brio 1624; Madrid. Lozoya del Valle, El Chaparral, 17 Jun 2012, *Lara*, MAUAM-Brio 3300; Miraflores, road to La Morcuera, 16 Sep 1990, *Lara*, MAUAM-Brio 1640; Balearic Islands. Mallorca, 14 Abr 1999, *Cano*, MAUAM-Brio 3328; Murcia. El Selva, 2 May 1998, *Sánchez Moya*, MUB 15070; Tarragona. Roquetas, Macizo dels Ports, 22 Jul 1995, *Vergara & Lara*, MAUAM-Brio 1657; **Tunisia:** Montes Aïn-Drahim, Jbel Bir, 22 Mar 2005, *Lara & San Miguel*, MAUAM-Brio 2401; **Turkey:** Adana. Anti-Taurus, from Kozan to Feke, 13 Jul 2006, *Albertos, Estébanez, Garilleti & Medina*, MAUAM-Brio 3103; Antalya. Taurus Lycicos, Ak Daĝlan, Seki-Ceylan, 19 Jul 2006, *Albertos, Estébanez, Garilleti & Medina*, MAUAM-Brio 3127; Taurus Lycicos, Bey Daĝlan, way up to Termessos, 18 Jul 2006, *Albertos, Estébanez, Garilleti & Medina*, MAUAM-Brio 3119; Taurus Lycicos, Bey Daĝlan, way up to Termessos, 18 Jul 2006, *Albertos, Estébanez, Garilleti & Medina*,

MAUAM-Brio 3125; Taurus Psidiánicos, Çalpete, Köprü river, 18 Jul 2006, *Albertos, Estébanez, Garilleti & Medina*, MAUAM-Brio 3115; Aydin. Samsun Dag, Dilek National Park, Olukludere Kanyon, 22 Jul 2006, *Albertos, Estébanez, Garilleti & Medina*, MAUAM-Brio 3129 [**12:** *KT862270 / KT862358 / KT862301 / KT862330*]; **USA:** California. Riverside Co., San Bernardino National Forest, San Jacinto Mts., Hwy. 243, 16 Nov 2008, *Lara, Garilleti & Shevock*, MAUAM-Brio 3323 [**2:** *KT862273 / KT862361 / KT862304 / KT862333*]; Riverside Co., San Bernardino National Forest, San Jacinto Mts., north of Vista Grande Forest Service, 31 Dec 2012, *Lara, Mazimpaka & Vigalondo*, MAUAM-Brio 3321, 3322 [**1:** *KT862274 / KT862362 / KT862305 / KT862334*]; San Diego Co., Cleveland National Forest, Laguna Mt. Recreation Area, 29 Dec 2012, *Lara, Mazimpaka & Vigalondo*, MAUAM-Brio 3314, 3315 [**4:** *KT862271 / KT862359 / KT862302 / KT862331*]; San Diego Co., Cleveland National Forest, near Julian, 30 Dec 2012, *Lara, Mazimpaka & Vigalondo*, MAUAM-Brio 3316, 3317, 3318, 3319 [**3:** *KT862272 / KT862360 / KT862303 / KT862332*]; San Diego Co., Cuyamaca, Rancho State Park, Paso Picacho Campground, 15 Nov 2008, *Lara, Garilleti & Shevock*, MAUAM-Brio 3324.

Additional samples used for DNA extraction

Macrocoma lycopodioides. **South Africa:** Western Cape. Cape Town, Table Mountain, Nursery Valley, *Lara & San Miguel*, MAUAM-Brio 2953 [*KT862258 / KT862346 / KT862288 / KT862317*].

Orthotrichum affine. **USA:** California. Shasta Co., along Soda Creek, south of Dunsmuir Norris & Hillyard, MAUAM-Brio 4447 [*KT862276 / KT862365* / - / -]; **Spain:** Jaen. Martos, Sierra de Víboras *Lara*, MAUAM-Brio 4448 [*KT862277 / KT862363 / KT862306 / KT862335*]; **Turkey:** Artvin. Road between Sariğol and Barhal, NW de Yusufeli, *Lara, Medina & Mazimpaka*, MAUAM-Brio 4449 [*KT862278 / KT862364 / KT862307 / KT862336*].

Orthotrichum laevigatum. **Spain:** Madrid. Rascafría, Peñalara Natural Park, lakes of Los Llanos de Peñalara, *Albertos & Lara*, MAUAM-Brio 4461 [*KT862279 / KT862366 / KT862308 / KT862337*]; **USA:** Nevada. Humboldt Co. Humboldt National Forest, Santa Rosa Mountains, *Lara, Garilleti, Shevock & Albertos*, MAUAM-Brio 3297 [*KT862280 / KT862367 / KT862309 / KT862338*].

Orthotrichum lyellii. **USA:** California. San Diego Co., Cleveland National Forest, *Lara, Mazimpaka & Vigalondo*, MAUAM-Brio 4451 [*KT862282 / KT862368 / KT862310 / KT862339*].

Orthotrichum rupestre. **USA:** California. Mariposa Co., Yosemite National Park, Merced River, *Lara, Garilleti & Albertos*, MAUAM-Brio 4453 [*KT862283 / KT862369 / KT862311 / KT862340*].

Orthotrichum speciosum. **Turkey:** Artvin. Road from Sariğolto Barhal, NW de Yusufeli, *Lara, Medina & Mazimpaka*, MAUAM-Brio 3061 [*KT862284 / KT862372 / KT862313 / KT862342*];

USA: California. Mariposa Co., Yosemite National Park, Merced River, *Lara, Garilleti & Albertos,* MAUAM-Brio 4452 [*KT862281 / KT862373 / KT862312 / KT862341*].

***Orthotrichum speciosum* var. *brevisetum.* Spain:** Jaén. Los Villares, road from Fuensanta to Valdepeñas, *Lara,* MAUAM-Brio 4425 [*KT862286 / KT862370 / KT862315 / KT862344*]; **Turkey:** 10 - 15 km from Arseky, *Bermejo & Martínez,* MAUAM-Brio 4450 [*KT862285 / KT862371 / KT862314 / KT862343*].

Orthotrichum striatum. **Turkey:** Antalya. Taurus Isáuricos, Geyik Daĝlan, Akseki, Irmasan Geçidi, *Albertos, Estébanez, Garilleti & Medina,* MAUAM-Brio 4446 [*KT862287 / KT862374 / KT862316 / KT862345*].

***Zygondon pentastichus.* Argentina:** Córdoba. Nequén, Villa La Angostura, Nahuel Huapi Lake, *Lara & San Miguel*, MAUAM-Brio 2981 [*KT862259 / KT862347 / KT862289 / KT862318*].

***Zygodon viridissimus.* England:** Lake District, Borrowdale valley, Derwent, *Lara,* MAUAM-Brio 2910 [*KT862260 / KT862348 / KT862290 / KT862319*].

Supplementary Material

Figure 3.1.S1. Beanplots of other gametophyte and sporophyte quantitative variables of *Orthotrichum acuminatum* for each geographical region. See Figure 4 for more information.

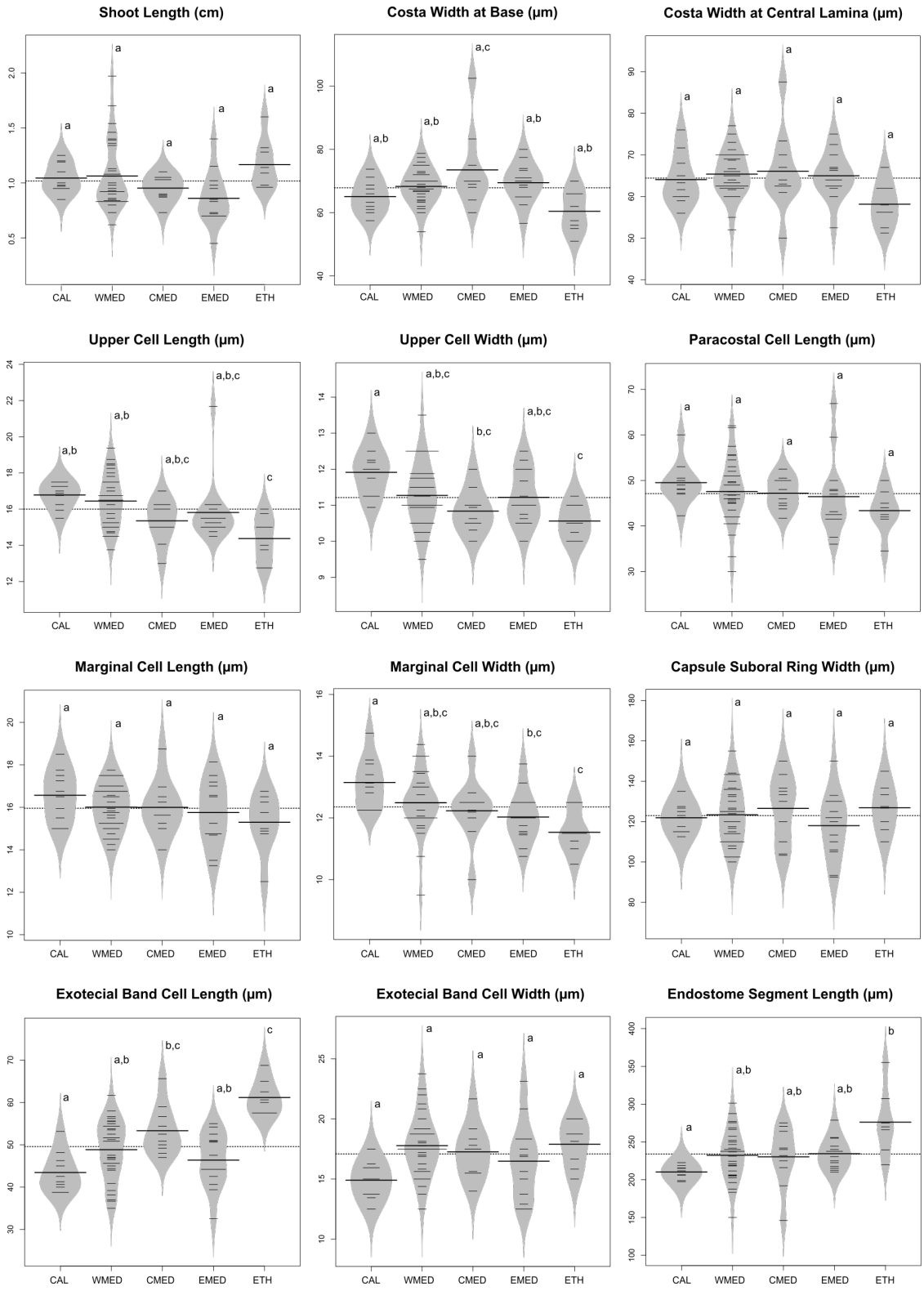

Table 3.1.S1. Summary of qualitative variation in *Orthotrichum acuminatum* according to the three main geographic areas analysed.

	California	**Mediterranean**	**Ethiopia**
Gametophyte			
Habit	Cushion	Cushion	Cushion
Colour	Light green to dark green	Light green to dark green	Light green to dark green
Stem section shape	Rounded-pentagonal	Rounded-pentagonal	Rounded-pentagonal
Axillary hairs (basal brown + hyaline cells)	1-2 + 3-4	1-2 + 3-4	1-2 + 3-4
Rhizoids position on stems	Lower parts, often ascending	Lower parts, occasionally ascending	Restricted to lower parts
Rhizoids colour	Brown-reddish or orange	Brown-reddish or orange	Brown-reddish or orange
Rhizoids papilosity	Smooth	Smooth	Smooth
Leaf position when dry	Appressed to erect	Appressed to erect	Appressed to erect
Leaf position when wet	Patent to spreading	Patent to spreading	Patent to spreading
Leaf section	Moderately keeled	Moderately keeled	Moderately keeled
Leaf shape	Lanceolate to ovate-lanceolate	Lanceolate to ovate-lanceolate	Lanceolate to ovate-lanceolate
Leaf apex shape in upper leaves	Acute to acuminate, sometimes asymetric	Acute to acuminate, sometimes asymetric	Acute to acuminate, sometimes asymetric
Perichaetial leaf apex shape	Acuminate to long acuminate or subulate	Acuminate to long acuminated or subulate	Acuminate to long acuminated or subulate
Leaf margin	Recurved from base to near apex	Recurved from base to near apex, sometimes not recurved at base	Recurved from base to near apex
Leaf margin near apex	Entire or slightly crenulate	Entire or slightly crenulate	Entire or slightly crenulate
Leaf margin strata	Monostrate	Monostrate	Monostrate
Leaf lamina strata	Entirely monostrate	Entirely monostrate	Entirely monostrate
Leaf lamina papillosity	0-1(2) low papillae per cell-side	0-1(2) low papillae per cell-side	0-1(2) low papillae per cell-side
Leaf costa distal end	Below apex to percurrent, rarely briefly excurrent	Below apex to percurrent, rarely briefly excurrent	Below apex to percurrent, rarely briefly excurrent
Leaf acumen cells	Mostly indifferentiated with a variable proportion of elongate to vermiculate cells	Mostly indifferentiated with a variable proportion of elongate to vermiculate cells	Mostly indifferentiated with a variable proportion of elongate to vermiculate cells

Table 3.1.S1. Continuation

	California	Mediterranean	Ethiopia
Sporophyte			
Vaginula hairs	Always present, few, often long	Occasionally present, few, short	Exceptionally present, few, short
Sporophyte foot penetration in the stem	Always	Always	Always
Capsule position	Immersed	Immersed	Immersed
Capsule body colour	Light yellow, brownish when old	Light yellow, brownish when old	Light yellow, brownish when old
Capsule suboral ring colour	Green, brown to orange when old	Green, brown to orange when old	Green, brown to orange when old
Capsule shape when dry and full	Mostly fusiform	Mostly fusiform	Mostly fusiform
Capsule shape when dry and empty	Fusiform to cylindrical	Fusiform to cylindrical	Fusiform to cylindrical
Capsule shape when wet	Mostly cylindrical	Mostly cylindrical	Mostly cylindrical
Capsule ribs when dry	Slightly prominent, usually more marked near mouth	Slightly prominent, usually more marked near mouth	Slightly prominent, usually more marked mouth
Capsule ribs when wet	Diffuse	Diffuse, sometimes obscure	Diffuse
Number of ribs/exothecial bands	8	8	8
Length of exothecial bands	Upper 1/3 of capsule	Upper 1/3 of capsule	Upper 1/3, occasionally to ca. 1/2 of cap
Exothecial band cell-rows	2-4	2-4	2-4
Exothecial cells colour	Almost hyaline, with yellow walls	Almost hyaline, with yellow walls	Almost hyaline, with yellow walls
Exothecial cells wall	Thin to slightly thickened	Thin to slightly thickened	Thin to slightly thickened
Exothecial cells shape	Rectangular to polygonal	Rectangular to polygonal	Rectangular to polygonal
Annulus	Present, hyaline to orange, (1)2 cell rows	Present, hyaline to orange, 1(2) cell rows	Present, hyaline to orange, 1(2) cell rows
Stomata position	Middle and upper part of the capsule	Middle and upper part of the capsule	Middle and upper part of the capsule
Type of stomata	Phaneroporous	Phaneroporous	Phaneroporous

Table 3.1.S1. Continuation

	California	Mediterranean	Ethiopia
Sporophyte			
Exostome teeth	Rudimentary, only basal part developed	Rudimentary, only basal part developed	Rudimentary, only basal part developed
Exostome teeth general aspect	Fragmentary and hidden to complete and protruding beyond the mouth	Fragmentary and hidden to complete and protruding beyond the mouth	Fragmentary and hidden to complete and protruding beyond the mouth
Exostome teeth cell number (PPL)	(2)3-5(6)	(1)2-3(5)	2-3
Exostome teeh colour (wet/dry)	Hyaline/white	Hyaline/white	Hyaline/white
Exostome OPL	Usually absent, if present openly papillose	Usually absent, if present openly papillose	Usually absent, if present openly papillos
Exostome PPL	Openly to densely ornamented with stout papillae	Openly to densely ornamented with stout papillae	Openly to densely ornamented with stout
Endostome segments	8	8	8
Endostome colour (wet/dry)	Hyaline/white	Hyaline/white	Hyaline/white
Endostome segments position (dry)	Incurved closing the mouth	Incurved closing the mouth	Incurved closing the mouth
Endostome segments general aspect	Irregular margins, (1)2 rows of cells	Irregular margins, (1)2 rows of cells	Irregular margins, (1)2 rows of cells
Endostomial connective membrane	Absent, sometimes fragments present	Absent, sometimes fragments present	Absent, sometimes fragments present
Endostome PPL ornamentation	Very fine papillae, dispersed	Very fine papillae, dispersed	Very fine papillae, dispersed
Endostome IPL ornamentation	Stout papillae densely disposed	Stout papillae, densely disposed	Stout papillae densely disposed
Lid shape	Plane to convex, rostrate	Plane to convex, rostrate	Plane to convex, rostrate
Lid colour / basal ring color	Light yellow / green	Light yellow / green	Light yellow / green
Calyptra shape	Oblong-conic	Oblong-conic	Oblong-conic
Calyptra hairiness	Moderately hairy	Moderately hairy, sometimes almost naked	Moderately hairy
Calyptra hairs	1–2-seriate, usually almost smooth	1–2-seriate, usually almost smooth	1–2-seriate, usually almost smooth
Calyptra capsule coverage	ca. 2/3 of the capsule	ca. 2/3 of the capsule	ca. 2/3 of the capsule
Spore ornamentation	Fine and densely papillose	Fine and densely papillose	Fine and densely papillose
Polysety	Common	Occasional	Not detected

Table 3.1.S2. Classificatory matrix from discriminant function analysis (DFA) for the three main geographical regions, showing the number of specimens from *Orthotrichum acuminatum* allocated to each group based on original and cross-validated grouped cases.

			Predicted Group Membership			
		Groups	California	Mediterranean	Ethiopia	Total
Original	Count	California	10	0	0	10
		Mediterranean	0	56	0	56
		Ethiopia	0	0	8	8
	%	California	100	0	0	100
		Mediterranean	0	100	0	100
		Ethiopia	0	0	100	100
Cross-validated	Count	California	8	2.0	0.0	10.0
		Mediterranean	3	51	2	56
		Ethiopia	0	1	7	8
	%	California	80	20	0	100
		Mediterranean	5	91	4	100
		Ethiopia	0	13	88	100

100.0% of original grouped cases correctly classified. 89.2% of cross-validated grouped cases correctly classified.

Table 3.1.S3. Classificatory matrix from discriminant function analysis (DFA) for five groups considering the three Mediterranean sub-regions, showing the number of specimens from *Orthotrichum acuminatum* allocated to each group based on original and cross-validated grouped cases.

			Predicted Group Membership					
		Groups	California	Western Mediterranean	Central Mediterranean	Eastern Mediterranean	Ethiopia	Total
Original	Count	California	10	0	0	0	0	10
		Western Mediterranean	0	29	2	1	0	32
		Central Mediterranean	0	1	9	0	0	10
		Eastern Mediterranean	0	4	1	9	0	14
		Ethiopia	0	0	0	0	8	8
	%	California	100	0	0	0	0	100
		Western Mediterranean	0	90.6	6.3	3.1	0	100
		Central Mediterranean	0	10	90	0	0	100
		Eastern Mediterranean	0	28.6	7	64.3	0	100
		Ethiopia	0	0	0	0	100	100
Cross-validated	Count	California	8	2	0	0	0	10
		Western Mediterranean	3	17	5	6	1	32
		Central Mediterranean	0	5	3	2	0	10
		Eastern Mediterranean	0	5	1	7	1	14
		Ethiopia	0	1	0	0	7	8
	%	California	80	20	0	0	0	100
		Western Mediterranean	9	53.1	15.6	18.8	3.1	100
		Central Mediterranean	0	50	30	20	0	100
		Eastern Mediterranean	0	35.7	7	50	7.1	100
		Ethiopia	0	12.5	0	0	87.5	100

87.8% of original grouped cases correctly classified. 56.8% of cross-validated grouped cases correctly classified.

Chapter

3.2

Searching for new molecular markers

Comparing three complete mitochondrial genomes of the moss genus *Orthotrichum* Hedw.

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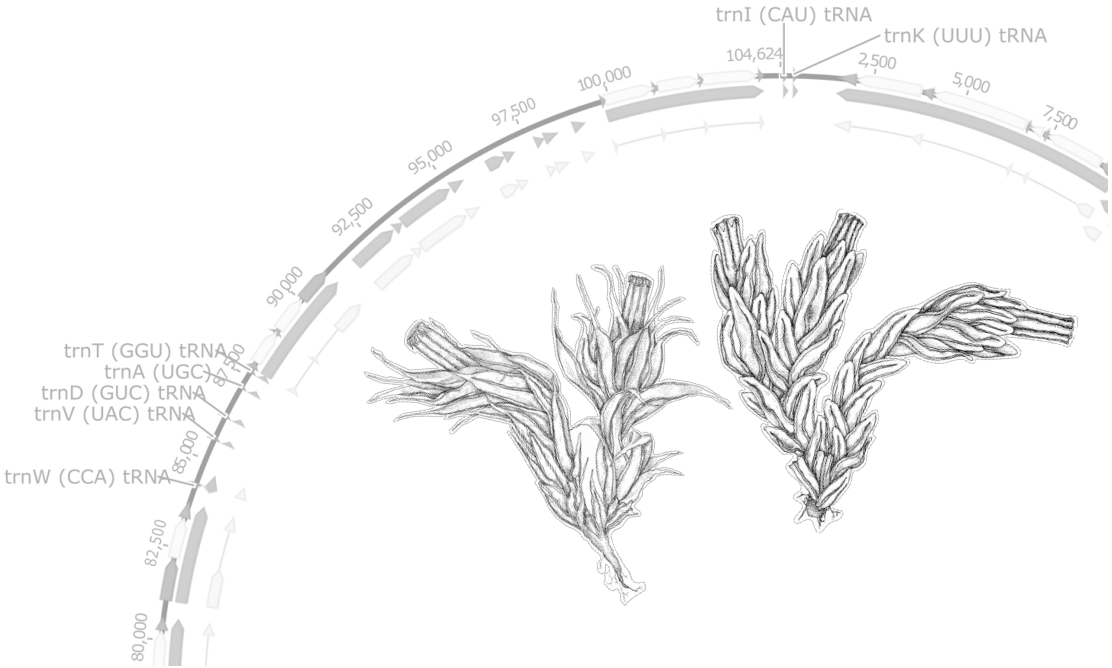
Published in *Mitochondrial DNA Part B* (2016) 1:1, 179-181



Abstract

Here we present a comparative analysis of the mitochondrial genome of three representatives of *Orthotrichum* Hedw. (Bryophyta): two populations of *O. diaphanum* and one of the related species *O. macrocephalum*. Their mt genomes share the same genic content and gene order, and are furthermore structurally identical to those of other arthrodontous mosses. The mitogenome of the allopatric samples of *O. diaphanum* differ in 0.1% of their sequence, with protein coding genes holding five mutations, including two non-synonymous changes. The divergence between the mitogenomes of the two species, *O. diaphanum* and *O. macrocephalum*, is 0.4%. Within a broader sampling of the Orthotrichoideae, patterns of genome divergence are consistent with phylogenetic relationships.

The genus *Orthotrichum* is one of the most species-rich moss genera, with 163 species (Medina R. *et al.,* 2013). *Orthotrichum diaphanum* Brid. and *O. macrocephalum* F. Lara, Garilleti and Mazimpaka are two related epiphytic species of section Diaphana Vitt. (Lara *et al.,* 1994) with distinct but overlapping geographic distributions: *O. diaphanum* occurs throughout the Western Palearctic–Western Nearctic, whereas *O. macrocephalum* is restricted to the Mediterranean areas in the Northern Hemisphere.

The number of moss mitochondrial (mt) genomes announced has dramatically increased in recent years (Liu *et al.,* 2011, 2014; Sawicki *et al.,* 2014; Alonso *et al.,* 2015; Sawicki *et al.,* 2015), but only one study (Lewis *et al.,* 2016) has targeted the mt genome of several conspecific populations. We sought to assess the types and distribution of substitutions between the genome from two populations of *O. diaphanum* and between this species and the related O. *macrocephalum.*

Multiple gametophytes and/or sporophytes were collected from three samples: *Orthotrichum diaphanum* #1 (MAUAM-Brio 4559; Spain, 6°45'34.2"N 5°22'07.3"W), *O. diaphanum* #2 (MAUAM-Brio 4560; Germany, 10, 52º18'14.8"N 12º59'11.3"E) and *O. macrocephalum* (MAUAM-Brio 4561; Spain, Hoyo de Manzanares, 40º37'15"N 3º54'48.25"W). Total DNA was extracted using the NucleoSpin plant II® Midi kit (Macherey Nagel GmbH & Co. KG, Düren, Germany). Three genomic DNA libraries were prepared using the Nextera kit (Illumina, CA), and then multiplexed and sequenced on an Illumina MiSeq instrument using a 600-cycle v3 sequencing kit (Illumina, CA). Following the filtering and trimming of the reads with Trimmomatic v0.33 (Bolger *et al.,* 2014), the resulting paired-end reads were de novo assembled using CLC Genomics Workbench v6.5 (CLC Bio, Aarhus, Denmark) with the default assembly parameters. All de novo contigs were blasted with CLC BLAST tool to the *O. stellatum* Brid. mt genome (NC_024522, Liu *et al.,* 2014). A single mt contig was obtained for *O. diaphanum* #1 (total contigs = 27,499; N50 = 1,444 bp) and *O. diaphanum* #2 (total contigs = 65,946; N50 = 1,773 bp) whereas for *O. macrocephalum,* two contigs were recovered (total contigs = 25,937; N50 = 1,869 bp). All contigs were first visually inspected for unexpected drops in depth, and then aligned against the reference and imported to Geneious (Biomatters Ltd., Auckland, New Zealand). Low-depth areas in a contig or gaps between contigs were confirmed or closed through a series of reference alignments and assemblies following (Fučíková *et al.,* 2014). These gaps sequences were verified by PCR and Sanger sequencing. The complete mt genomes were annotated in Geneious 7.1.2 using extracted annotations from *O. stellatum.* Coding regions were checked with an ExPASy translation tool (Gasteiger *et al.,* 2003), and annotations manually corrected. Exon and intron boundaries were further confirmed against orthologs from other species.

To confirm the phylogenetic identity of the samples, we inferred their relationships with other 13 moss species publicly available, including members of the *Orthotrichaceae* (see Fig.1 for GenBank accession numbers). Protein-coding genes sequences were aligned using the progressive Mauve algorithm (Darling *et al.*, 2004) in Geneious, in order to perform phylogenetic analyses under maximum likelihood and Bayesian inference.

The total length for the mt genome of *O. diaphanum* #1 (KT_373970) is 104,756 bp (106x coverage), *O. diaphanum* #2 (KT_823697) 104,744 bp (163x coverage), and *O. macrocephalum* (KT_823696) 104,624 bp (60x coverage). The GC content of the three samples is the same as for other published *Orthotrichaceae* (i.e., 39.8%; Liu *et al.*, 2014; Sawicki *et al.*, 2014, 2015). The three mt genomes contain the same set of genes (i.e., 40 protein-coding, 24 tRNA, and 3 rRNA genes) organized in the exact same order as in other Orthotrichaceae and most of other mosses (Liu *et al.*, 2014; Sawicki *et al.*, 2014; Sawicki Jakub *et al.*, 2015; Yoon *et al.*, 2016).

The phylogenetic inferences (Fig. 3.2.1) are congruent with the phylogenetic structure among moss genera (Liu *et al.*, 2014; Young-Jun *et al.*, 2015). *Orthotrichum* is known to be polyphyletic, which is confirmed here with species of *Orthotrichum* with superficial stomata more closely related to *Ulota* D. Mohr than to species with immersed stomata (Goffinet *et al.*, 2004).

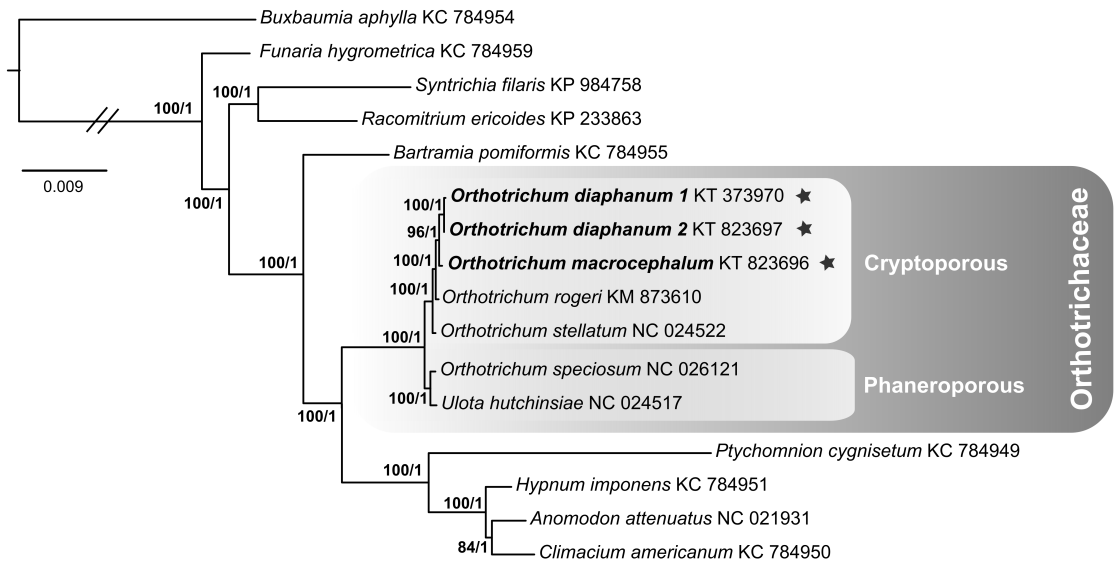


Figure 3.2.1. Majority-rule consensus tree of the Bayesian inference analyses of 40 mitochondrial protein coding genes, showing the phylogenomic affinities of Orthotrichum diaphanum and O. macrocephalum (indicated with stars). Bootstrap values under maximum likelihood (>50) followed by posterior probabilities (>0.95) of Bayesian inference are indicated near the corresponding branch. GenBank accession numbers follow taxon names. Scale bar represents substitutions per site rate.

The two mt genomes of *O. diaphanum* differ in 68 bp (i.e., 0.1%), and when *O. macrocephalum* is added, the number of variable sites increases to 398 bp (i.e., 0.4%). Across Orthotrichum species with immersed stomata (cryptoporous; *O. diaphanum*, *O. macrocephalum*, *O. rogeri* Brid. and *O. stellatum*) the mitogenomes differ in 1,241 bp (i.e., 1.2%), whereas the two taxa with superficial stomata (phaneroporous; *O. speciosum* Nees and *Ulota hutchinsiae* (Sm.) Hammar) differ in 605 bp (i.e., 0.6%). The divergence between species of *Orthotrichum* with immersed and superficial stomata is 1,903 bp (i.e., 1.8%), which is higher than between *O. speciosum* and *Ulota*, as would be expected from their phylogenetic relationship (Goffinet *et al.*, 2004, Fig. 6.1). Within the *Orthotrichoideae*, the mitogenome varies in 2,288 sites (i.e., 2.1%). Compared to the only other moss subfamily for which more than two mitogenomes have been assembled, the *Orthotrichoideae* exhibit more variation than the three species of *Funarioideae* (i.e., 1.5%; Liu *et al.*, 2014).

Within *O. diaphanum*, the variable sites are relatively scarce and widely dispersed along the mt genome. Sixty-three substitutions occur within non-coding regions, and five (three transitions and two transversions) within protein coding regions. Among the latter, two substitutions result in non-synonymous changes (i.e., in the rps1 gene: A<–>C, 3rd codon position of the 211th codon, Asparagine to Lysine; ccmFN gene: A<–>G, 1st codon position of the 175th codon, Asparagine to Aspartic acid). The only concentration of mutations occurs in the cox1 group II intron cox1i1064g2, which holds two mononucleotide substitutions, one 6 bp indel, and either five or three TATAT microsatellite repeats in *O. diaphanum* #1 and #2, respectively. The alignment of both *O. diaphanum* and *O. macrocephalum* mitogenomes, and that of all *Orthotrichaceae*, reveals noticeable interspecific variation, most of it in non-coding regions, such as cox1 and cox2 group II introns, but also within coding regions such as ccmFN gene. Those regions could potentially be evaluated as new markers for phylogenetic analyses within this moss family.

Acknowledgements. The authors thank Dr. Karolina Fučíková (UConn) for providing helpful comments on the assemblies' methods and Dr. Rafael Medina for his advice and assistance during the development of this study.

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Chapter

3.3

Orthotrichum shevockii

The long journey of *Orthotrichum shevockii* (Orthotrichaceae, Musci): from California to Macaronesia

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Pending of publication

Abstract

Biogeography and taxonomy are intimately integrated disciplines. In order to know where a species grows, its origin, its colonization routes and its evolutionary history, first it is necessary to correctly establish species boundaries. Populations of an unknown moss were found on the Canary Islands (Tenerife), which resembled two different endemic species from California: *Orthotrichum shevockii* and *O. kellmanii*. Integrative taxonomic analyses reveal that *O. kellmanii* actually corresponds to *O. shevockii*, which results to be a morphologically variable taxon. Besides, morphological and molecular results confirm that the populations from Tenerife also belong to *O. shevockii*, exposing an interesting case of bryophytes disjunction between western North America and Macaronesia. Divergence time estimation and ancestral area reconstruction analyses support the hypothesis of long-distance dispersal to explain this disjunction, and establish the Californian origin of the populations of *O. shevockii* from Tenerife. These results agree with the ideas that the Macaronesian cryptogamic flora has a different origin from that of the angiosperms, and that long-distance dispersal can contribute to explain the low rates of bryophyte endemism existing in these islands.

Introduction

After Wegener's plate tectonics theory, ancient fragmentation was long considered to be the main process that explains common distribution patterns in plant biogeography (Raven & Axelrod, 1974), while dispersal was seen as a random and irrelevant process (Cowie & Holland, 2006). However, in the last decades, molecular tools and the development of dating and divergence time estimations have pointed to dispersal as a key process for explaining current species distribution (Renner, 2004; Queiroz, 2005; Kadereit & Baldwin, 2012; Christenhusz & Chase, 2013; Vargas *et al.*, 2014). In the case of volcanic oceanic islands that have originated without a connection to a continental landmass, dispersal is considered to play a fundamental role in the generation of biodiversity and biogeographical patterns (Cowie & Holland, 2006; Sanmartín *et al.*, 2008; Baldwin & Wagner, 2010; Gillespie *et al.*, 2012; Alsos *et al.*, 2015; Vargas *et al.*, 2015). For Macaronesian islands (including the Canary Islands, Azores, Madeira and Cabo Verde archipelagos), it has been suggested that endemic bryophytes exhibit a completely different evolutionary origin from angiosperms due to their different dispersal capabilities, since ancestors of endemic bryophytes seem to come from more distant areas (Carine *et al.*, 2004; Vanderpoorten *et al.*, 2011). This agrees with the greater distribution ranges of bryophytes with respect to the tracheophytes, which is also attributed to their higher dispersal capabilities (Vanderpoorten & Goffinet, 2009). In many cases, these ranges involve intercontinental disjunctions at species level, while in vascular plants these only occur at generic level (Vanderpoorten & Goffinet, 2009; Medina N.G. *et al.*, 2011).

Few recent studies support the traditional hypothesis of vicariance through ancient fragmentation for the origin of wide disjunct distributions in bryophytes (McDaniel & Shaw, 2003; Heinrichs *et al.*, 2006; Hedenäs, 2008). However, there is a growing evidence of long distance dispersal (LDD) as the mechanism shaping different trans-continental and trans-oceanic bryophyte distributions (Muñoz *et al.*, 2004; Piñeiro *et al.*, 2012; Lewis *et al.*, 2014; Pisa *et al.*, 2014; Sun *et al.*, 2014; Kyrkjeeide *et al.*, 2016; Scheben *et al.*, 2016; Carter *et al.*, 2017). This also applies for taxa present in Macaronesia (Vanderpoorten *et al.*, 2008; Patiño *et al.*, 2013b; Patiño & Vanderpoorten, 2015; Pisa *et al.*, 2015). However, processes like incomplete lineage sorting, slow evolution rate (Szövényi *et al.*, 2008; Stenøien *et al.*, 2011; Draper *et al.*, 2015), or cryptic speciation (for review see Shaw, 2001; Heinrichs *et*

al., 2009), can also be contributing to create apparently wide and disjunct patterns in bryophytes. In other cases, this situation can be the consequence of an incomplete taxonomical knowledge (i.e. Medina R. *et al.,* 2012, 2013). All this suggests that an accurate species delimitation is a necessary first step in order to legitimate distribution patterns, and to perform biogeographic analyses in bryophytes, and the integrative perspective has proved to be especially useful for this (Medina R. *et al.,* 2012, 2013; Renner *et al.,* 2013; Hedenäs *et al.,* 2014; Draper *et al.*, 2015; Heinrichs *et al.,* 2015; Caparrós *et al.,* 2016; Carter *et al.*, 2017; Sim-Sim *et al*., 2017).

In the course of recent field surveys in Tenerife Island (Canary Islands), several saxicolous populations of an unknown *Orthotrichum* Hedw. were found in the area of the Teide volcanic cone known as *Las Cañadas del Teide*, at altitudes around 2100 m.a.s.l., growing in crevices of volcanic rocks and walls. A preliminary morphological examination of these specimens revealed that their main characteristics differed from any *Orthotrichum* species known in the Mediterranean and North Atlantic areas. Surprisingly, these populations resembled two different species from western North America: 1) *Orthotrichum shevockii* Lewinsky-Haapasaari & D.H. Norris, a saxicolous moss described from two localities of dry mountain areas in southern Sierra, California, between 1150 and 1600 m.a.s.l., restricted to granitic rock outcrops where it grows in ceilings of large boulders; and 2) *Orthotrichum kellmanii* D.H.Norris, Shevock & Goffinet, another saxicolous species, known from just a few localities of central California coastal mountains subjected to the influence of summer fogs from the Pacific Ocean, where it grows on sandstone rock outcrops in chaparral areas at altitudes around 650 m.a.s.l. These two similar species seem to mainly differ in gametophytic traits. *Orthotrichum shevockii* is characterized as having leaves with bi- to tristratose margins and highly papillose leaf cells (Lewinsky-Haapasaari & Norris, 1998). *Orthotrichum kellmanii* was described as developing leaves with completely bistratose lamina, although Norris *et al.* (2004) based its differentiation mainly in the presence of heterophyllous leaves (different leaf shape in reproductive and vegetative axes), and a weakly cladocarpous growth. Meanwhile, the new specimens from the Canary Islands have leaves with a very variable extent of bistratosity among different individuals, from completely bistratose leaf laminae to bistratosity restricted to the leaf margins.

The bryophyte flora of Macaronesia is one of the best known among oceanic island regions worldwide. However, the knowledge of its diversity and rates of endemism is still

incomplete, as suggested by the increasing number of recent descriptions and re-circumscriptions of species for this region based on molecular data (e.g. Aigoin *et al.*, 2009; Hutsemékers *et al.*, 2012; Hedenäs *et al.*, 2014; Draper *et al.*, 2015; Vanderpoorten *et al.*, 2015; Sim-Sim *et al.*, 2017). Given this background, doubts arose about the true nature of the populations discovered on the Canary Islands, which suggested the need for an in-depth study. Therefore, we address here the following questions: 1) which is the identity of the new moss found on the Canary Islands; 2) which are the taxonomical relationships between the Canarian moss and the Californian *O. shevockii* and *O. kellmanii*; and 3) which is the evolutionary and biogeographical history of these mosses? To infer these central issues, we use an integrative taxonomic approach combining morphological analyses, phylogenetic inferences, molecular dating and estimation of ancestral ranges.

Material and methods

Sample design

The material for this study includes the gatherings made on Tenerife, herbarium specimens of *Orthotrichum shevockii* and *O. kellmanii* from UC, CAS, CONN and NY herbaria (including type materials), and specimens obtained during specific collecting campaigns throughout several Californian mountain ranges and neighbouring regions of western Nevada. Specimens were selected in order to represent the geographical distribution and ecological range of the species. Thirty samples were included in the morphological analyses: 9 from Tenerife and 21 from California (Fig. 3.3.1, Appendix). Considering the availability and quality of the material, for molecular analyses a subset of 16 samples that were representative of the morphological diversity and geographic distribution of the samples was selected (Fig. 3.3.1, Table 3.3.S1). We also included as ingroup specimens of other *Orthotrichum* species, comprising several ones inhabiting the western coast of North America and the Canary Islands, some of them being endemic of these areas (Lewinsky-Haapasaari & Norris, 1998; Medina R. *et al.*, 2012; Patiño *et al.*, 2013b), to provide a phylogenetic frame for the assessment of the monophyly of the group. Three species of *Lewinskya* F.Lara, Garilleti & Goffinet, one of *Macrocoma* (Hornsch. *ex* Müll.Hal.) Grout, one of *Nyholmiella* Holmen & E.Warncke, and two of *Zygodon* Hook. & Taylor were selected as outgroup, bringing a total number of 66 samples (see Table 3.3.S1 for voucher information and GenBank accession numbers).

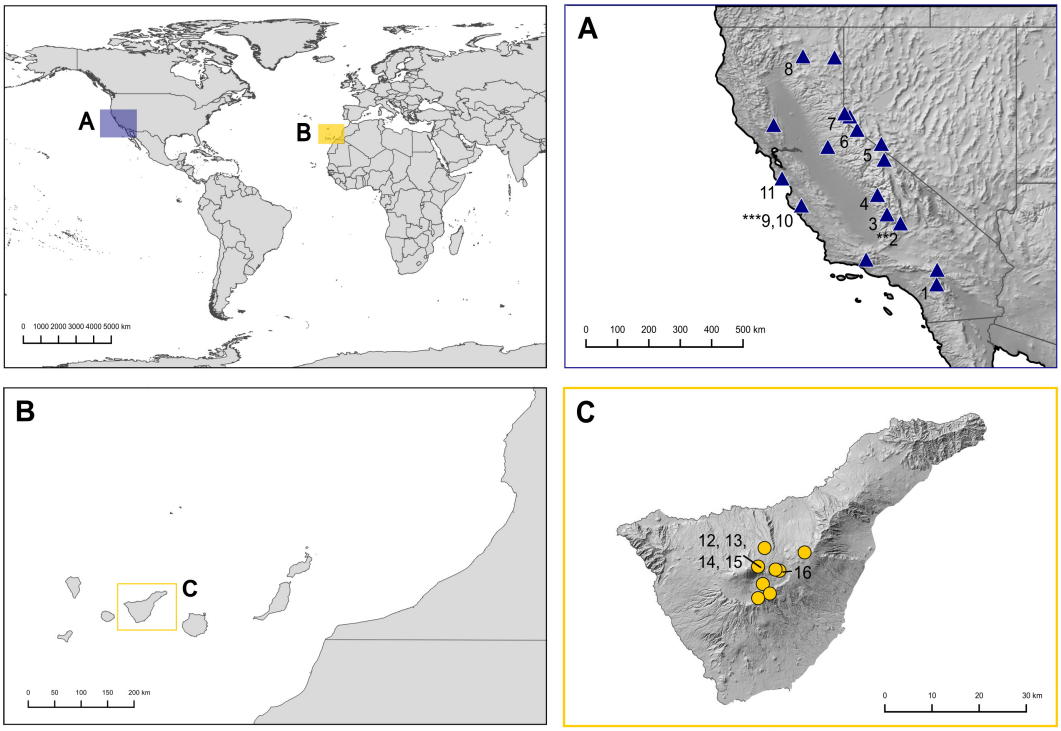

Figure 3.3.1. Procedence of the studied specimens of *Orthotrichum shevockii* from California and Nevada (A) and the Canary Islands (B), Tenerife (C). Numbers indicate specimens included both in morphometric and phylogenetic analyses (see Appendix and Table 3.3.S1). **= original locality of O. *shevockii*, *** = original locality of *O. kellmanii*.

Morphological analyses

An intensive morphological analysis was conducted on the 30 selected specimens to assess the differences between the Californian ones ascribable to either *Orthotrichum shevockii* or *O. kellmanii*, and those from Tenerife. A set of morphological characters, both qualitative and quantitative, were selected and studied according to our previous experience in Orthotrichaceae (Lara *et al.*, 2009; Medina R. *et al.*, 2012, 2013; Lara & Garilleti, 2014; Vigalondo *et al.*, 2016).

Considered qualitative traits of the gametophyte include plant habit, several leaf characters such as leaf shape, margins, and lamina bistratosity, and cell papillosity, as well as calyptra and vaginula hairiness. Sporophyte characters are usually of great diagnostic value in the genus (Vitt 1973, Lewinsky 1993), and we focused the study on operculum shape, capsule shape, exothecial bands structure, stomata position, and structure and ornamentation of the peristome.

For quantitative morphometric analyses, 16 characters were selected (Table 3.3.1). Measurements and construction of the data set protocol follows Vigalondo *et al.* (2016). To detect a possible unknown underlying structure within the dataset, an exploratory multivariate analysis was performed (principal component analysis, PCA). A correlation matrix was used in the PCA to scale the morphological variables, and only principal components (PCs) accounting for more than 10% of the variance were considered in the results. Univariate analysis of variance (ANOVA) was conducted to assess the homogeneity of variances for each of the 16 quantitative variables for California and Tenerife specimens. Multivariate analyses were run twice: (i) discarding samples with missing values; and (ii) replacing missing values by the mean value of each character. Results from both approaches were congruent (results not shown), so to avoid reducing the sampling size, for the final analyses we used the data set with missing values replaced by the mean. Descriptive statistics were finally computed for all quantitative variables, considering populations from Tenerife and California separately. The results were summarized in the form of beanplot graphs (Kampstra, 2008), representing the empirical density shape, mean, and all individual observations for each of the two evaluated geographical groups. All statistical analyses were implemented using R v.3.3.1 (R Core Team, 2016).

DNA extraction and sequencing

DNA was extracted from apices of stems and branches from dried herbarium specimens. Total DNA was extracted using the DNeasy® Plant Mini Kit for DNA isolation from plant tissue (Qiagen). We selected four loci previously used for phylogenetic reconstructions of *Orthotrichum* (Medina R. *et al.*, 2012; Vigalondo *et al.*, 2016): three chloroplast loci, namely *atp*B-*rbc*L, *rps*4, and *trn*L-F, and the nuclear internal transcribed spacer II (ITS2). The primer pairs used for each locus were *atb*1/*rbc*L1 (Chiang *et al.*, 1998), *rps*A/*trna*S (Nadot *et al.*, 1994; Souza-Chies *et al.*, 1997), *trn*C/*trn*F (Taberlet *et al.*, 1991) and *ITS2F/ITS2R* (Fiedorow *et al.*, 1998).

Double-stranded DNA templates were prepared by PCR, which was performed using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech Inc.) in a final reaction volume of 25 μL according to the manufacturer's instructions. PCR amplifications of *atp*B-*rbc*L, *rps*4, and *trn*L-F were performed using the protocol described in Medina R. *et al.* (2012), while the ITS2 protocol followed Vigalondo *et al.* (2016) approach. PCR products were

purified using Exo/SAP protocol (Thermo Fisher Scientific, Spain). Samples were incubated with 1 µL of Exo1 enzyme and 4 µL of FastAP following the manufacturer's instructions. Cleaned PCR products were sequenced by Macrogen (www.macrogen.com). All new sequences were deposited in GenBank (see Table 3.3.S1).

Table 3.3.1. Quantitative characters evaluated for *Orthotrichum shevockii* and results of quantitative morphometric analyses of.

Character	*Orthotrichum shevockii*		ANOVA	PC1	PC2	PC3
	Western North America (California)	Canary Islands (Tenerife)				
Gametophyte						
Shoot length [1]	0,49 ± 0,13 [0,3-0,81]	0,55 ± 0,16 [0,39-0,8]	0,990	0,277	-0,272	0,082
Upper leaf length [2]	2,32 ± 0,3 [1,77-3,02]	2,37 ± 0,26 [1,81-2,65]	0,153	**0,329**	0,272	-0,020
Upper leaf width [2]	0,59 ± 0,1 [0,41-0,78]	0,66 ± 0,11 [0,44-0,79]	2,212	**0,372**	0,016	0,072
Perichaetial leaf length [2]	2,88 ± 0,45 [2,18-4,08]	2,98 ± 0,21 [2,58-3,27]	0,306	**0,376**	0,166	0,081
Perichaetial leaf width [2]	0,73 ± 0,12 [0,52-0,97]	0,84 ± 0,1 [0,7-1,04]	4,833*	**0,389**	-0,004	0,042
Sporophyte						
Vaginula length	425,36 ± 60,49 [320-510]	400,56 ± 76,46 [325-550]	0,866	0,155	0,010	0,054
Seta length	536,08 ± 66,33 [425-650]	526,55 ± 68,57 [443,33-650]	0,097	0,175	-0,047	0,057
Capsule length [1]	1,47 ± 0,14 [1,23-1,71]	1,45 ± 0,11 [1,25-1,62]	0,172	0,261	-0,059	**0,314**
Capsule neck length	419,54 ± 56,39 [320-543,33]	417,83 ± 44,41 [380-525]	0,007	0,191	**0,425**	0,053
Exotecial band width	130,35 ± 20,65 [87,5-170]	145,94 ± 17,87 [122-174]	3,869	0,031	**-0,374**	0,132
Exotecial band cell length	36,74 ± 6,95 [25,5-55]	36,96 ± 4,03 [31,5-42,5]	0,007	0,213	0,244	-0,181
Exotecial band cell width	27,52 ± 3,18 [22,5-35,5]	25,33 ± 5,52 [18,5-34]	1,896	-0,135	**-0,331**	0,217
Endostome segment length	222,97 ± 49,62 [150-319,38]	202,72 ± 43,38 [106-246,25]	1,155	0,190	**-0,361**	0,151
Exostome teeh length	250,48 ± 59,46 [154,17-343,75]	260,27 ± 30,62 [202,5-290,83]	0,216	0,297	**-0,356**	-0,036
Spore length	12,43 ± 0,91 [10,31-13,75]	11,86 ± 0,76 [10,63-13,13]	2,722	-0,166	0,231	**0,574**
Spore width	12 ± 0,96 [10,31-13,75]	11,37 ± 0,44 [10,63-12,19]	3,512	-0,091	0,133	**0,648**

Descriptive statistics for quantitative characters are represented for each of the geographical groups of *Orthotrichum shevockii* (mean ± SD [range]); all measurements are in µm except those with [1] = cm and [2] = mm. ANOVA F statistic and significance level (* ≤ 0.05) for each variable and each region are given. PCA component loadings for each original variable are represented. In bold, variables with the highest loadings for each component. Percent of total variance explained for first component (PC1) = 33.96 %, second component (PC2) = 13.62 % and third component (PC3) = 12.4%; see Fig. 3.3.5 and 3.3.S1.

Phylogenetic and dating analyses

Nucleotide sequence contigs were edited and assembled for each DNA region using Geneious 7.1.2 (http://www.geneious.com, Kearse *et al.*, 2012) and PhyDE v.0.9971 (Müller *et al.*, 2006). Sequences were aligned manually and trimmed at the ends. Regions of ambiguous or incomplete data were identified with GBlocks (Talavera & Castresana, 2007) and excluded from subsequent analyses.

Phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI). The best-fitting substitution models for each matrix locus were inferred under the Bayesian Information Criterion (BIC) in jModelTest v.2.1.3 (Darriba *et al.,* 2012). Maximum likelihood analyses were run with RAxML 8 (Stamatakis, 2014), and the best ML tree was selected from 100 iterations and its support was assessed with 1000 replicates of bootstrap resampling under the ML criterion. Bayesian phylogenetic analyses were carried out using MrBayes v.3.2.1 (Ronquist *et al.,* 2012). The Markov chain Monte Carlo (MCMC) simulation was run for 2 to 5 million of generations with two runs and four chains, sampling trees and parameters every 1000 generations. After checking that stationarity had been reached (i.e.: the average standard deviation of split frequencies remained below 0.01 for the last 10,000 generations), posterior probabilities (PP) were estimated from the 50% majority-rule consensus trees after a burn-in of 25% of the starting trees. The resulting trees for both ML and BI analyses were plotted using FigTree v.1.4.2 (Rambout, 2012).

Insertions and deletions (indels) in non-coding regions are sometimes difficult to assess (Kelchner, 2000) and can lead to ambiguous alignments. To determine the effect of their inclusion, phylogenetic information from indels was coded as an adjacent block with the program SeqState (Müller, 2012) using the simple indel coding method (Simmons & Ochoterena, 2000). The analyses were performed with and without codified indels with the same parameters indicated above, using model F81 for the indel partition in MrBayes, as recommended by Ronquist *et al.* (2011).

All independent gene data sets were combined in a single concatenated matrix, as no incongruences were identified in branches supported with posterior probability ≥ 0.95 and bootstrap support ≥ 85 when each gene was analysed separately (data not shown). Sequences with some loci information missing were omitted. The resulting concatenated data set was analysed in PartitionFinder (Lanfear *et al.,* 2012) to select the best partitioning scheme and nucleotide substitution model, using the greedy algorithm with linked branch lengths under the BIC criterion. Three partitions were defined: ITS2 (HKY+G), *rps*4 (HKY+G) and the combined *atp*B-*rbc*L and *trn*L-F (GTR+G).

Divergence times were estimated using BEAST 1.8.0 (Drummond *et al.,* 2012). Because the inclusion of identical sequences in dating analysis results in many zero length branches at the tip of the tree and can cause the model to overpartition the data set (Reid & Carstens,

2012), we reduced the data set to haplotypes (30 sequences) using DnaSP 5.10.1 (Librado & Rozas, 2009). For all the analyses, clock and tree models were linked across partitions, and models of substitution were unlinked across loci. Both strict and uncorrelated log-normal relaxed clocks were tested under two different speciation tree models: Yule and birth–death process. In the absence of fossil records of *Orthotrichum*, an absolute nucleotide substitution rate (mean = 4.453E-4 and stdev = 1.773E-6 substitutions/site/million of years) was incorporated to the *ucld.mean* parameter in BEAST, and sampled from a log-normal distribution according to the results of relaxed-clock analyses across the Moss Tree of Life (Laenen *et al.*, 2014). All BEAST analyses were run for four independent chains of 80 million generations each, sampling every 10 thousand generations and their convergence was assessed by confirming that all parameters had reached stationarity and sufficient effective sample sizes (> 200) in all converged runs using Tracer v1.6 (Rambaut *et al.*, 2014). The best model was selected through Marginal likelihoods estimates (MLEs) that were assessed using path-sampling (PS, Lartillot *et al.*, 2006) and stepping-stone (SS, Xie *et al.*, 2011) methods. The resulted MLEs were averaged across replicate runs to generate a single PS and SS value for each model. The obtained MLEs for all hypothesis were ranked, and Bayes factors were then calculated. In this study the Birth-Death process model performed best (Table 3.3.S2). After discarding the burn-in steps, tree files from the four independent runs of the selected model were combined using LogCombiner 1.8 (Drummond *et al.*, 2012) and the resulting maximum clade credibility (MCC) tree was summarized in TreeAnnotator 1.8 (Drummond *et al.*, 2012) and viewed in FigTree v.1.4.2 (Rambout, 2012).

Ancestral area estimation

We defined six geographical areas based on the main aim of the present study of inferring the historical biogeography of *Orthotrichum shevockii*, and also considering the whole distribution of the rest of the ingroup species: western North America (W), eastern North America (E), Caribbean, Central America and South America (N), Europe (U, including the Mediterranean and North Africa), Macaronesia (M), and Asia (A). We used the time-calibrated MCC tree obtained from BEAST but removing the outgroups, to perform ancestral area estimations across the *Orthotrichum* ingroup with the R package BioGeoBEARS (BioGeography with Bayesian Evolutionary Analysis in R Scripts, Matzke, 2014). We applied six different biogeographical models (DEC, DEC + J, DIVALIKE,

DIVALIKE + J, BAYAREA, BAYAREA + J) under a maximum likelihood framework, and compared how well they fit the data using the Akaike Information Criterion (AIC) (Matzke, 2013, 2014). The three main models allow for different biogeographical possibilities and include two free parameters (*d*= dispersal, *e*=extinction); the free parameter "+ J" corresponds to founder-event speciation, allowing long-distance dispersal events.

Results

Morphological analyses

Based on morphological traits, herbarium materials and recently collected specimens from California and Nevada could not be clearly separated and ascribed to neither *Orthotrichum shevockii* nor *O. kellmanii*. All the specimens, including the analysed type material of both taxa, share an ample number of basic qualitative characters: 1) plants acrocarpic, forming dark cushions or tufts; 2) leaves erect-appressed when dry (Fig. 3.3.2), spreading when moist; 3) leaves mostly ligulate at stems basal part, lanceolate upwards (Fig. 3.3.3); 4) costa ending bellow apex; 5) cladautoicous sexual condition; 6) vaginula naked, rarely with scarce long hairs; 7) calyptra oblong-conic, hairy (Fig.3.3.2); 8) capsule immersed to shortly emergent, short cylindric to urceolate when dry and empty, strongly 8 ribbed (Fig. 3.3.2); 9) exothecial bands broad, differentiated in the entire length of the urn (Fig. 3.3.4); 10) exostome of 8 teeth pairs easily and variably splitting (Fig. 3.3.4); 11) endostome of 8 segments, filiform, hyaline, almost as long as teeth (Fig. 3.3.4); 12) operculum slightly convex, short rostrate, with a thin basal orange rim (Fig. 3.3.2); 13) spores small (≤15 μm in diameter), coarsely papillose. Interestingly, all samples share some further details that are very uncommon in genus *Orthotrichum*: 1) the stomata appear always restricted to the capsule neck, sometimes reaching the limit with the seta or the base of the urn (Fig. 3.3.4A, I); 2) the exostome teeth are usually lacunose, showing lacunae in both the external (OPL) and internal (PPL) layers around the median line, and sometimes also appear within the teeth cell areas (Fig. 3.3.4B, J, K); and 3) the endostome external layer (PPL) is frequently ornamented with oblique or vertical lines (Fig. 3.3.4D, L).

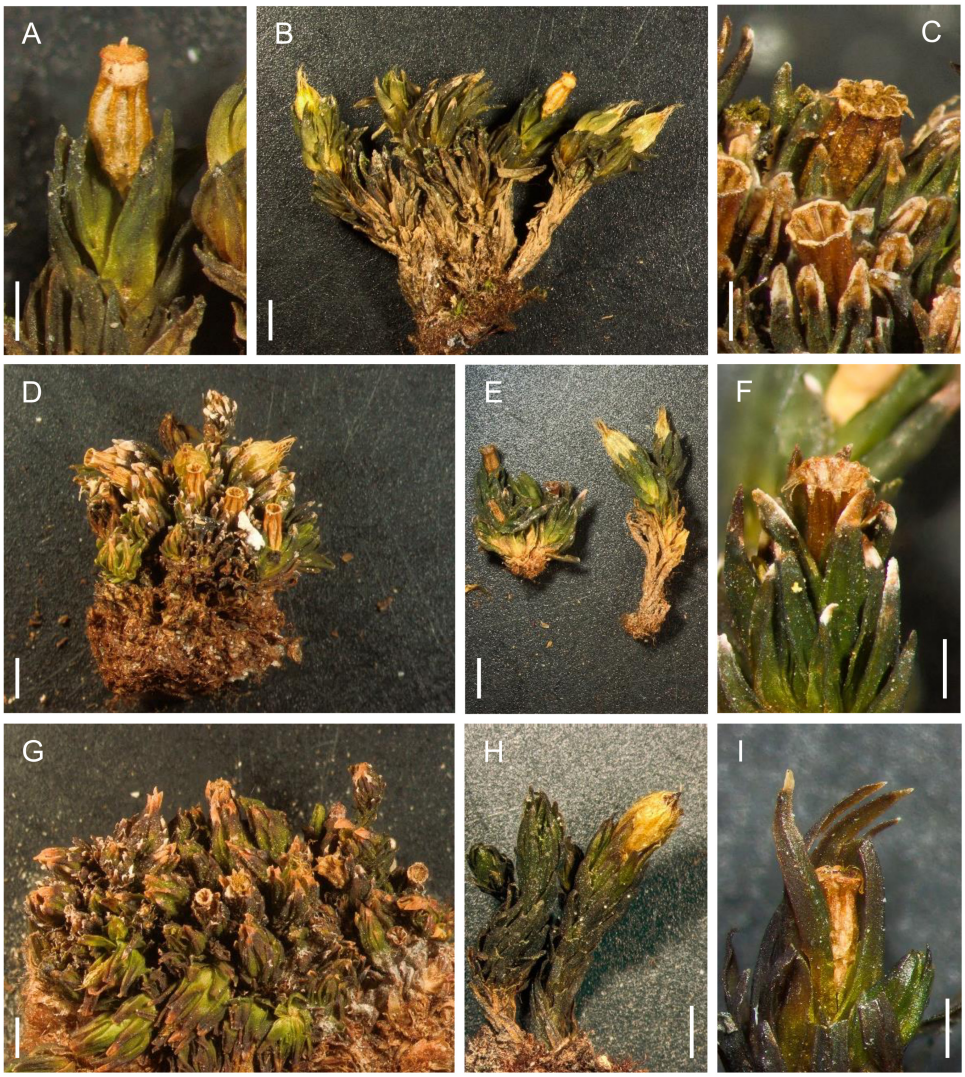

Figure 3.3.2. A-D: *Orthotrichum shevockii* from California; E-G: *O. shevockii* from Tenerife; H-I: *O. kellmanii* (=*O. shevockii*) from California. A, C, F, I= capsule detail; B, D, E, G, H= habit; G, two different habits from the same voucher. Scale bars: A, C, E, I = 0.5 mm, B, D, F, G, H = 1 mm — A-B, Shevock 13404 (CAS 958716, paratype), C, *Shevock 21948* (CAS 1040048); D, *Shevock 21802* (UC 1754431); F, *Losada-Lima, León & Díaz s.n.* (TFCBry 15904); E-G, *Losada-Lima s.n.* (TFCBry 17428); H, *Shevock 32935* (CAS s.n.); I, *Shevock* (NY 1140598).

Other qualitative characters exhibit broad variation among and within localities, and frequently even among stems from the same cushion. Concerning gametophyte traits, variation greatly affects the constitution of leaves. Leaf margins are typically bistratose but sometimes they can be 3-4 cells thick and exceptionally unistratose in most of their length (Fig. 3.3.3A). In parallel, leaf lamina is in many cases predominantly or completely bistratose in its upper 1/2-2/3 part, but frequently it is also only partially bistratose; in the latter cases leaf lamina can have sparse bistratose bands in its upper half or, rarely, only

small bistratose strands or patches restricted to the apex (Fig. 3.3.3A). Lamina leaf papillosity is also very variable, having the cells 2(3-4) papillae in each side. Moreover, papillae can be prominent, simple or bifurcate, or in other cases short or even negligible. Papillosity is in a great extent related to leaf thickness: bistratose leaves have lamina cells with low papillae or almost smooth, while leaves only bistratose at margins or with bistratose strands in the lamina show high and bifurcate papillae. Finally, upper leaves, both vegetative and perichaetial, can be broadly to narrowly lanceolate, and their apices are usually acute, although sometimes perichaetial leaves are shortly acuminate (Fig. 3.3.3). Plant habit also aries, since commonly cushions or tufts are short and dense, but in some cases they appear fairly longer and looser (Fig. 3.3.2).

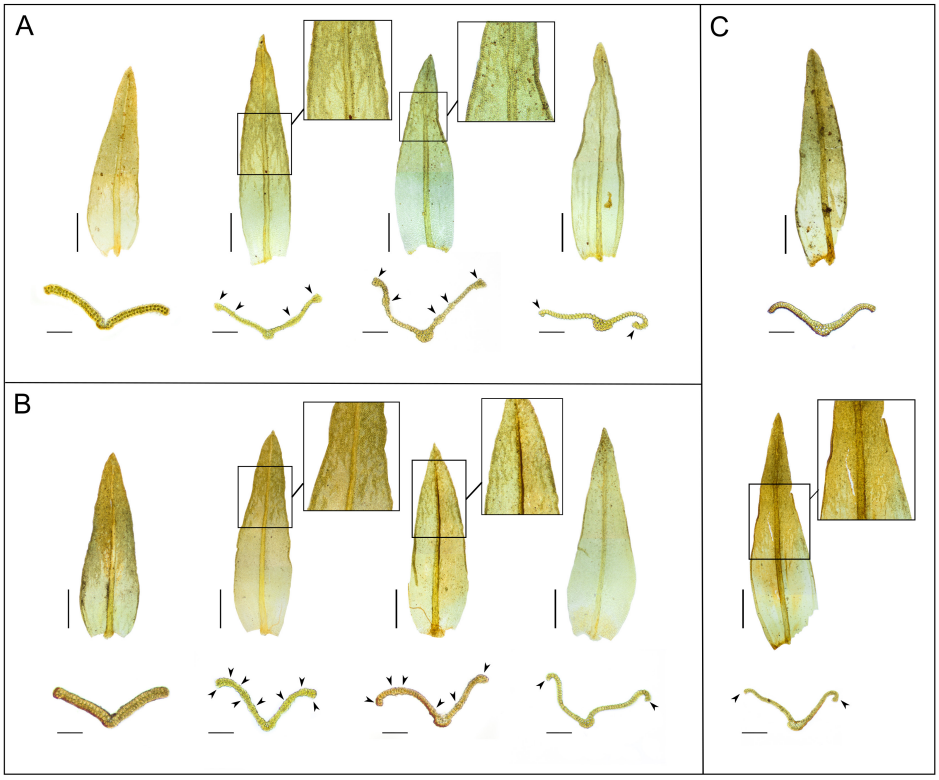

Figure 3.3.3. Leaf thickness variation shown in the entire leaf and leaf cross sections. A: *Orthotrichum shevockii* from western North America; B: *O. shevockii* from Tenerife, C: *O. kellmanii* (=*O. shevockii*) from California. A-B: from left to right: bistratose leaf (except base), leaf with bistratose upper part and bistratose bands, leaf with dispersed bistratose bands in the upper part, leaf almost unistratose with bistratose patches around the apex. C: top: bistratose leaf (except base), bottom: leaf with bistratose upper part and bistratose bands. Each leaf belongs to different samples. Cross sections belong to a different leaf of the same individual. Arrow heads indicate bistratose strands or margins, in C they indicate the tristratose margins. Scale bars: leaves = 0.5 mm, cross sections = 100 µm. — A: *Shevock 16754 & Anderson* (UC 1754230), *Lara et al. s.n.* (MAUAM-Brio 3289), *Shevock 13404 & York* (CAS 958716, paratype of *O. shevockii*), *Shevock 21802* (UC 1754431); B: *J.M.B., J.G.M. & J.L.P s.n.*, (TFC Bry 15957), *Losada-Lima s.n.* (TFCBry 17428), *Losada-Lima, León & Díaz s.n.* (TFC Bry 15861), *Losada-Lima, León & Díaz s.n.* (TFC Bry 15904); C: *Shevock 32935* (NY 1140598).

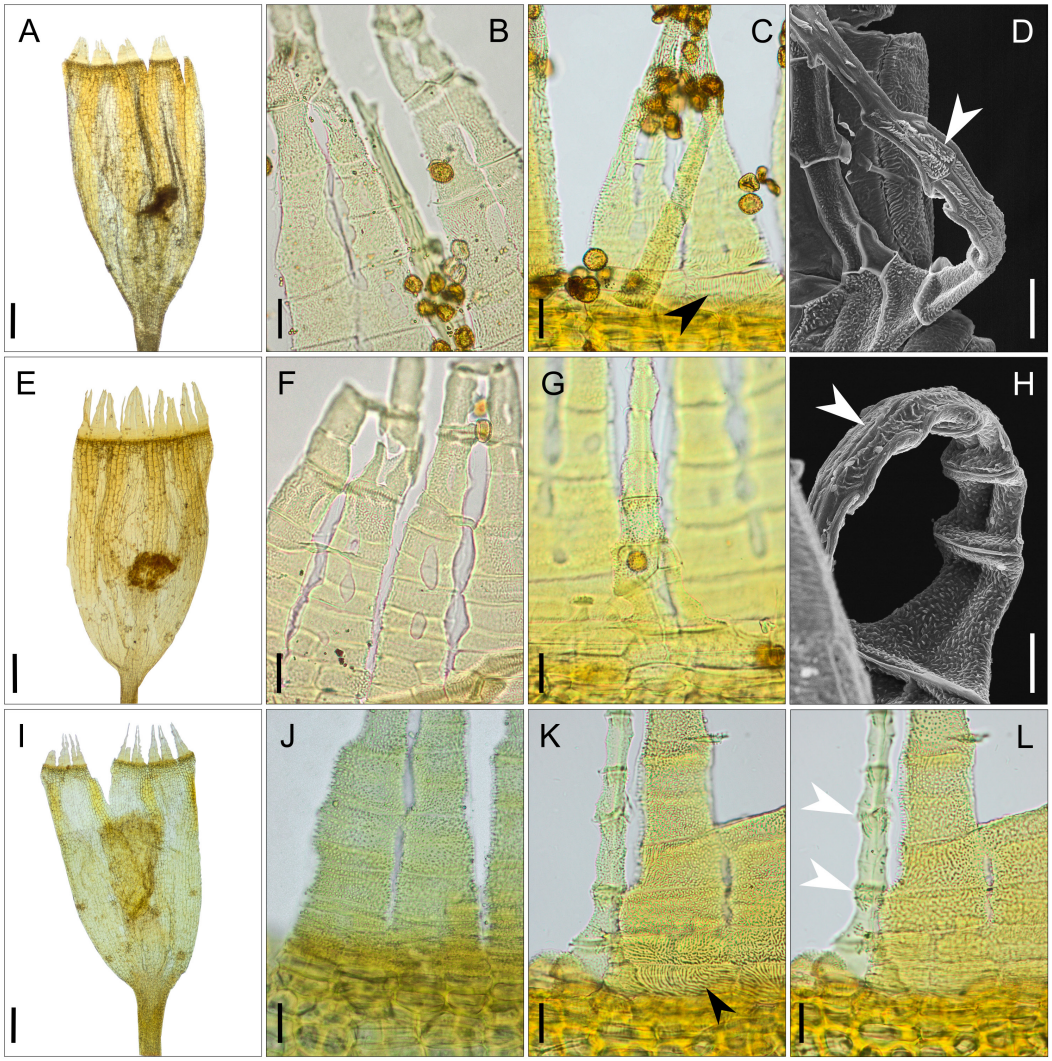

Figure 3.3.4. Capsule and peristome ornamentation. A-D: *Orthotrichum shevockii* from western North America; E-H: *O. shevockii* from Tenerife; I-K: *O. kellmanii* (=*O. shevockii*) from California. A, E, I: capsules with stomata restricted to the neck; B, F, J: exostome structure, showing the teeth lacunosity; C, G, K: endostome internal layer (IPL) papillose ornamentation; C, K: exostome internal layer (PPL), black arrows indicate striae at base; D, H, L: endostome external layer (PPL) ornamentation; white arrows indicate lines, sometimes forming plaques. Scale bars: A, E, I= 200 µm; B-D, F-G, J-L = 20 µm; H = 10 µm. — A, *Lara et al. s.n.* (MAUAM-Brio 3289); B, D, *Shevock 13404* (CAS 958716, paratype), C, *Shevock 21802* (UC 1754431); E, *Losada-Lima s.n.* (TFC Bry 15567); F, *Losada-Lima, León & Díaz s.n.* (TFCBry 15904); G, *Losada-Lima s.n.* (TFC Bry 17406); H, *Losada-Lima, León & Díaz s.n.* (TFC Bry 15952); I-J, *Shevock 32935* (CAS s.n.); K-L, *Shevock 32935* (NY 1140598).

As for the sporophytic traits, ornamentation of the different components of the peristome is noticeably variable. Exostome OPL consists at the basal part in a reticulum where transverse lines are usually more apparent, and it is covered by a variable proportion of papillae; in contrast, mid and upper parts of teeth have a denser ornamentation with a predominance of tall (occasionally low) papillae or, more rarely, vertical lines. Ornamentation of the inner surface (exostome PPL) can be papillose, reticulate, striate, or a

mix of both, and frequently it shows very well marked vertical striae at base (Fig. 3.3.4). Regarding the ornamentation of the endostome, the external layer (PPL) can be smooth or variably ornamented with lines, sometimes densely grouped in plaques (Fig. 3.3.4). The endostome internal layer (IPL) is rugulose or papillose, with papillae sometimes densely disposed and variably prominent (Fig. 3.3.4). Finally, exothecial bands are formed by 4–8 isodiametric to rectangular cells, varying among samples. Exothecial bands usually extend along the whole urn length but occasionally they are differentiated only in the upper half (Fig. 3.3.4).

The isotype material examined of *Orthotrichum kellmanii* and two other samples originally ascribed to this species fit the variability encountered for the rest of the studied Californian samples. They exhibit all the above mentioned basic characteristics and also those signaled as very uncommon within the genus. The only particularities noticed for these three samples mainly concern the leaf constitution. As in other Californian samples, leaves are extensively bistratose, both in margins and lamina, but exceptionally show up to three layers of cells in areas neighbouring the nerve and near the apex (Fig. 3.3.3C). In parallel, perichaetial leaves are consistently shortly acuminate. Although most sporophytes show the typical constitution above described, in some capsules, exothecial bands are unusually weak, constituted by 3-6 cell rows and restricted to the upper part of the urn (Fig. 3.3.4I). None of the material examined shows a cladocarpous growth pattern as described in (Norris *et al.*, 2004).

Specimens from Tenerife share with those from California all the qualitative characters mentioned above, showing the same degree of variation for gametophytic (Figs. 3.3.2, 3.3.3) and sporophytic traits (Fig. 3.3.4). The only peculiarities observed affect the frequency of some leaves trait states. In Tenerife leaf lamina is more commonly partially bistratose, prevailing the leaves with bistratose margins and dispersed bistratose bands in the upper part of lamina. Despite this, leaf cell papillae are commonly short. Finally, perichaetial leaves are usually broadly lanceolate (Fig. 3.3.3).

Statistical analyses of morphological quantitative traits also show no differences among California and Tenerife specimens. In PCA analyses the three first principal components (PCs) accounted for 59.98% of the variance. The PCA biplot shows dispersion of samples along the represented space, and no geographical nor taxonomical grouping is revealed. The

specimens from California and Tenerife overlap in the whole space (Figs. 3.3.5, 3.3.S1). With respect to the specimens originally identified as *O. kellmanii*, only one of them appears separated in the positive extreme of PC1. The most important variables in each of the three PCs (loading values ≥ 0.3) do not have any specific meaning, except for PC1 that represents leaf size, since the most important variables are those related to perichaetial and upper leaf length and width (Table 3.3.1, Fig. 3.3.5). When variables are considered independently comparing California and Tenerife, ANOVA analysis only shows significant differences for one variable: perichaetial leaves width (Table 3.3.1, Fig. 3.3.6).

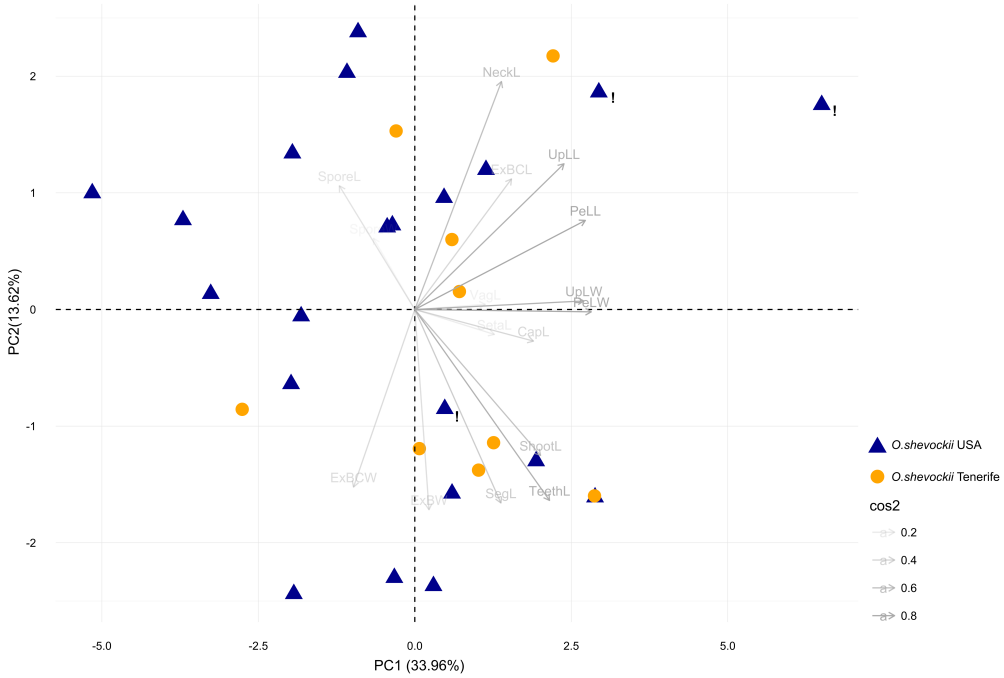

Figure 3.3.5. Results of the principal component analysis (PCA) representing the first two components. The percentage of variance explained by each component is given between brackets. Arrows represent the variables included in the analyses. cos2 represent the squared loadings for variables. ! = samples originally identified as *Orthotrichum kellmanii*.

Phylogenetic, dating and ancestral area reconstruction analyses

The resulting combined matrix of the four loci has a total length of 1715 bp, with 515 variable sites, of which 315 are potentially parsimony-informative. When including simple indel coding, we recovered trees with identical topology and no significant increase of support than those obtained when the indels were treated as missing data, so hereafter we will refer to the analyses and data without codified indels.

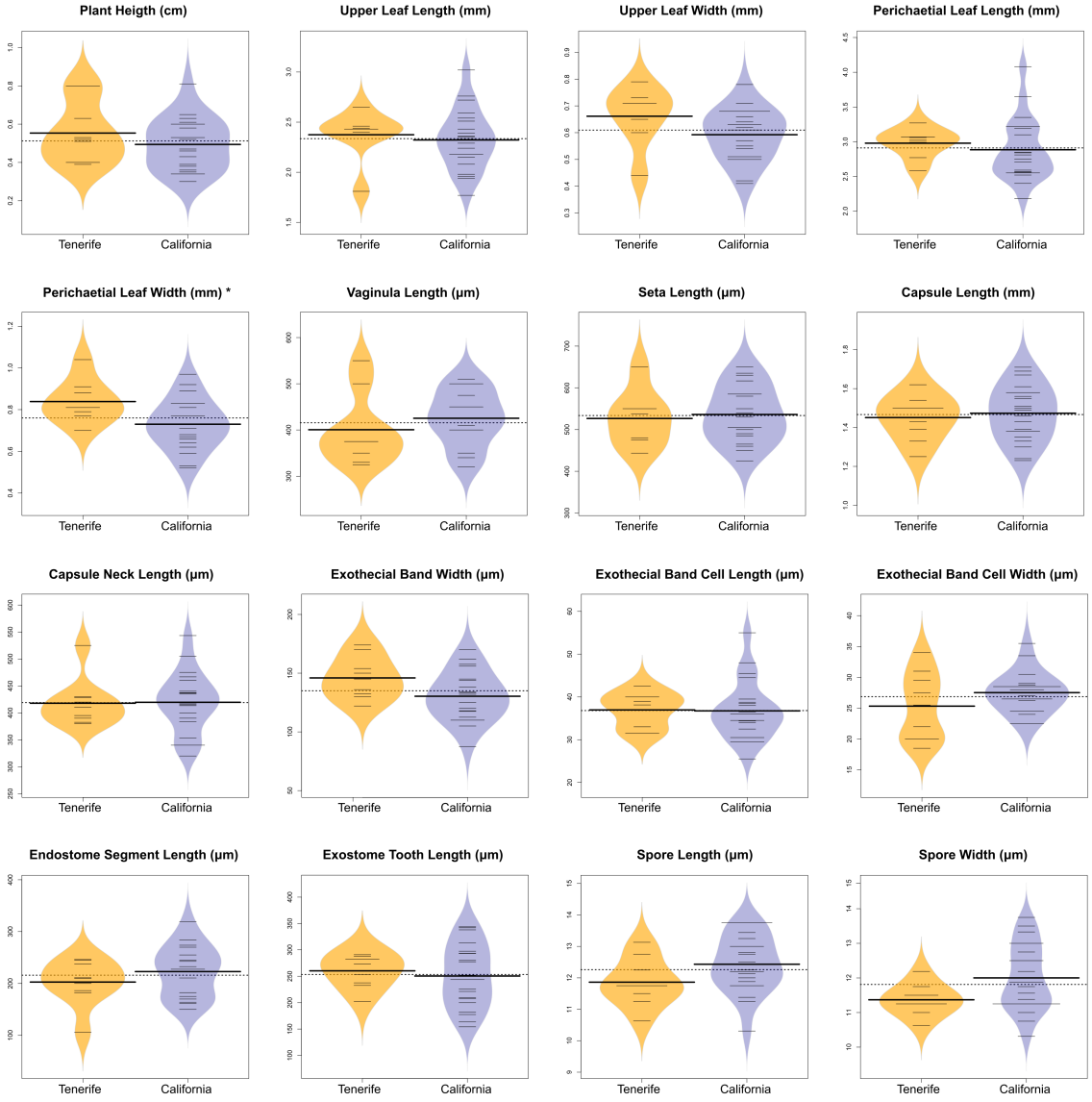

Figure 3.3.6. Beanplots of the studied quantitative variables of *Orthotrichum shevockii* from Tenerife (Canary Islands) and California (western North America, including *O. kellmanii* isotype materials). Individual observations are represented by small horizontal lines (in the case of multiple observations with the same values, the corresponding number of lines were merged), mean per group is shown by a bold long line and the mean for all data by a dotted line. Estimated density of the data distribution is displayed by the density shape in grey (for details see Kampstra, 2008). Stars indicate ANOVA significance values: * 0.05.

Phylogenetic analyses of ML and Bayesian inference resolved samples from California and Tenerife in the same monophyletic lineage with high support (Fig. 3.3.7; BS=75, PP=1.0). This one is embedded within a clade with a PP of 0.93, composed by taxa restricted to California and Nevada along with *Orthotrichum handiense* F.Lara, Garilleti & Mazimpaka from Fuerteventura (Canary Islands). Samples of *O. kellmanii* are also placed within the clade of *Orthotrichum shevockii* in BI and ML analyses.

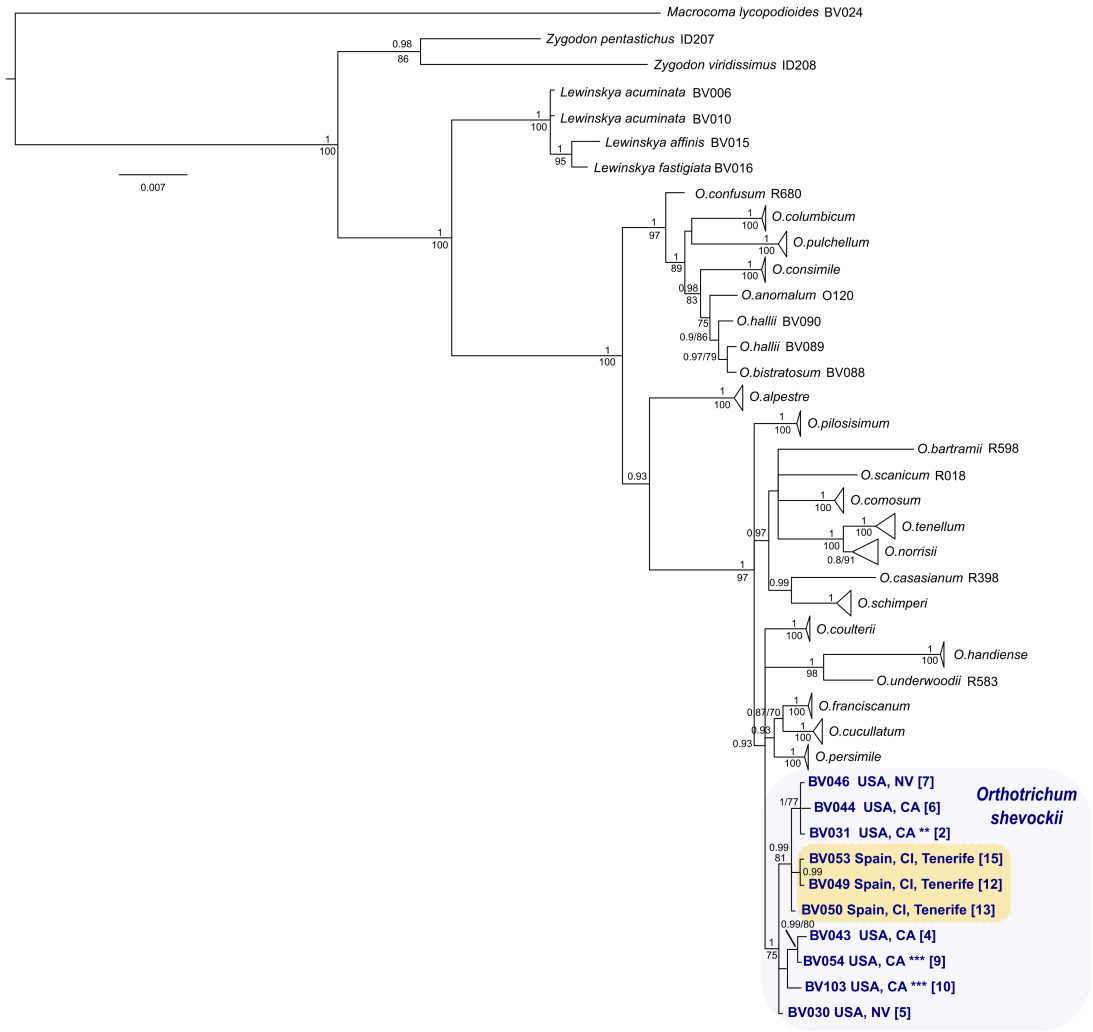

Figure 3.3.7. Majority-rule consensus tree obtained in the Bayesian analysis. Bayesian posterior probabilities (≥ 0.90) and maximum likelihood bootstrap values (≥ 70%) are shown above and below branches, respectively. Sequence labels of *Orthotrichum. shevockii* are followed by identification number, geographical origin, and number identification between brackets as in Figure 3.3.1 and Table 3.3.S1. **= paratype material of *O. shevockii*, *** = isotype material of *O. kellmanii*.

Dating analysis resolved that the split of *Orthotrichum shevockii* between California and Tenerife populations dated back to the early Miocene–Pliocene (2.74 Ma; 95% highest posterior density interval (HPD): 0.44–6.67 Ma, Fig.3.3.8 node A), and the common ancestor of the Californian clade dated back to 24.4 Ma (HPD: 10.9-31.45 Ma, Fig.3.3.8 node D). The best-fit model of ancestral area estimations, the DEC+J (Table 3.3.2), suggested that the present distribution of *O. shevockii* results from at least one long-distance dispersal event from western North America, which is supported by its inclusion in a highly supported western North American clade along with, as mentioned before, the Canary Island endemic *O. handiense* (Fig. 3.3.7).

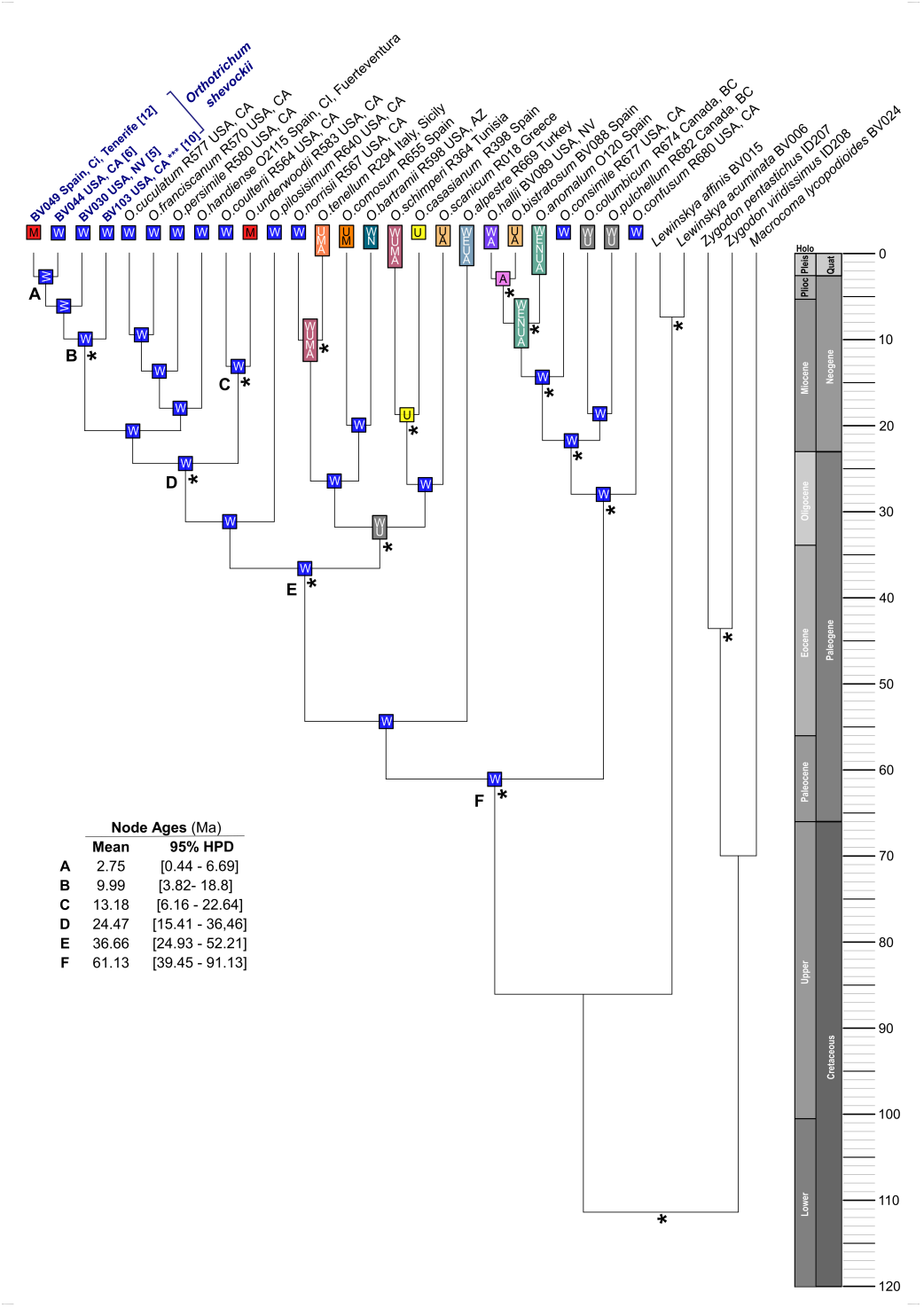

Figure 3.3.8. Molecular dating and biogeographic analyses. Maximum clade credibility tree from the relaxed molecular-clock analysis of the four loci in BEAST. Asterisks (*) at nodes refer to high supported nodes (PP >0.95). Sequence labels are followed by identification number and geographical origin. In the case of *Orthotrichum shevockii*, sequence labels are also followed by number identification between brackets as in Figure 3.3.1 and Table 3.3.S1 (*** = isotype material of *O. kellmanii*). For the *Orthotrichum* ingroup, letters in colour boxes correspond to the following ancestral areas or combination of areas according to the reconstructions based on the DEC + J model implemented in BioGeoBEARS: W = western North America; E = Eastern North America; N = Neotropics; M = Macaronesia; U = Europe; A = Asia. The probabilities of each biogeographical region are presented in Figure 3.3.S2.

Table 3.3.3. Performance, as assessed by log-likelihood (lnL) and the Akaike information criterion (AIC), of competing models of ancestral-area estimation (dispersal–extinction–cladogenesis, DEC; dispersal–vicariance analysis, DIVA; BayArea), as well as the same three models allowing for founder-event speciation (+J); *n*, number of parameters; *d*, rate of dispersal; *e*, rate of extinction; *j*, relative probability of founder-event speciation. The best model is highlighted in bold.

	ln*L*	***n***	***d***	***e***	***j***	**AIC**
DEC	-74,0978	2	0,0065	10^{-12}	0,0000	152,2
DEC+J	**-72,5518**	**3**	**0,0059**	**$2x10^{-9}$**	**0,0206**	**151,1**
DIVALIKE	-74,3172	2	0,0075	10^{-12}	0,0000	152,6
DIVALIKE+J	-74,1507	3	0,0072	10^{-12}	0,0077	154,3
BAYAREALIKE	-80,9334	2	0,0045	0,02	0,0000	165,9
BAYAREALIKE+J	-75,0962	3	0,0025	0,01	0,0321	156,2

Discussion

Taxonomic relationships of *Orthotrichum shevockii* and *O. kellmanii*

Species boundaries are sometimes difficult to establish, especially when there are subtle morphological or molecular differences among them (Bickford *et al.*, 2007). Recent studies in Orthotrichaceae (Caparrós *et al.*, 2016; Medina R. *et al.*, 2013, 2012) and other bryophyte groups (Renner *et al.*, 2013; Aranda *et al.*, 2014; Hedenäs *et al.*, 2014; Draper *et al.*, 2015) have evidenced the utility of integrative taxonomy in resolving cryptic species complexes and for species delimitation.

Our results show the lack of morphological or molecular differences that could support the consideration of *Orthotrichum shevockii* and *O. kellmanii* as separate species, based on the analysis of a significant number of samples and including type materials of both taxa. The morphological analyses revealed uniformity among these specimens for a great number of basic qualitative traits, but also for specific differential characters highlighted because of their exceptionality in the genus (Lewinsky, 1993; Medina R. *et al.*, 2012; Lara & Garilleti, 2014; Lara *et al.*, 2016): stomata restricted to the neck, exostome lacunose, and endostome PPL ornamented with lines or striae (Fig. 3.3.4). Moreover, quantitative traits do not reveal any structure among samples that could reflect a taxonomic differentiation (Fig. 3.3.5). In a complementary and concordant way, molecular results group the different samples ascribable to either of these two Californian species in the same well supported clade. Within this group, further segregation of samples seems to have no geographical, ecological or taxonomical meaning.

In our opinion, the previous consideration of *Orthotrichum shevockii* and *O. kellmanii* as two different species originally derives from two concurrent facts: the description of both taxa was made based on very few samples, corresponding each of them to extremes of the morphological variation of a highly variable moss with respect to some gametophytic traits. The firstly described, *O. shevockii* (Lewinsky-Haapasaari & Norris, 1998) was based on samples from two close inland localities of southern Californian mountains, whose specimens have leaves with bistratosity basically restricted to the margins. The second one, *O. kellmanii*, was described (Norris *et al.*, 2004) upon samples from two nearby coastal localities in which specimens show leaves with completely bistratose laminae and margins. Additionally, gametophores of these latter samples were considerably taller than usual, probably due to more favorable ecological conditions. In fact, these coastal localities are at low altitudes and receive more humidity due to the Pacific Ocean influence, with summer fogs, which could favor a greater development of the individuals. Specimens of this moss in such conditions form long sympodial gametophytic axes with both abundant basal short and ligulate leaves (identified as vegetative by Norris *et al.*, 2004), and upper leaves (from female axes), progressively larger and lanceolate. This could be the reason of Norris *et al.* (2004) to interpret the habit as weakly cladocarpic with extreme heterophylly. The development of shorter and somewhat different basal leaves, although rarely highlighted, is a common characteristic in *Orthotrichum* and related genera (see for example, Lara & Garilleti, 2014), whereas a true heterophylly related to male and female branches had been only reported for one European moss (Garilleti *et al.*, 2002).

Considering the above comments, it is clear that there are no morphological nor molecular characters to support the separation of *Orthotrichum kellmanii* and *O. shevockii*, and thereby we propose *O. kellmanii* to be synonymized with *O. shevockii*:

Orthotrichum shevockii Lewinsky-Haapasaari & D.H. Norris, Bryologist 101(3): 435. 1998.

= *Orthotrichum kellmanii* D.H.Norris, Shevock & Goffinet, Bryologist 107(2): 210. 2004. **syn. nov.**

Taxonomic position of Tenerife populations

Once the taxonomical concept of *Orthotrichum shevockii* is redefined, and its range of morphological variability in western North America is described, there is no doubt that the true identity of the new moss found in Tenerife (Canary Islands) also corresponds to *O. shevockii*. Specimens from this population are rather uniform in gametophyte and

sporophyte characteristics, and completely match the morphological variability encountered in North America. The fact that all Canarian and North American samples share some qualitative traits considered unusual within the genus is highly suggestive, reinforcing the morphological evidence on the conspecificity of these disjunct populations. In the particular case of *O. shevockii*, the stomata position, the exostome structure, and the endostome ornamentation (Fig. 3.3.4) could be considered as morphological markers, *i.e.* traits that allow elucidating the circumscription of conflicting samples to this species.

The molecular results obtained (Fig. 3.3.7) constitute the definitive support of the explained morphological evidence. Additionally, ecological aspects point in the same direction. In Tenerife *Orthotrichum shevockii* lives in arid regions at high altitudes, as a saxicolous moss that colonizes crevices, rock ceilings, and vertical faces on volcanic rocks. This is exactly the most frequent ecological situation where *O. shevockii* has been found in western North America, although lowland localities are also known, and granitic and sandstone rocks are likewise colonized.

The discovery of *Orthotrichum shevockii* as new for the Canary Islands rises to 14 the number of species of *Orthotrichum s.l.* known for this archipelago (González-Mancebo *et al.*, 2008; Ros *et al.*, 2013). This finding, together with the recent description of new species for Macaronesia (i.e. Draper *et al.*, 2015; Vanderpoorten *et al.*, 2015; Dirkse *et al.*, 2016; Patiño *et al.*, 2017; Sim-Sim *et al.* 2017), reveals that the knowledge of Macaronesia and, particularly the Canary Islands bryoflora is probably still incomplete (Vanderpoorten *et al.*, 2011). This evidences the need of increasing the effort in taxonomical studies and the importance to use integrative taxonomic approaches to reveal the real extant cryptogamic biodiversity of these archipelagos.

California – Macaronesia disjunction of *Orthotrichum shevockii*

Our results evidence that the distribution of *Orthotrichum shevockii* is disjunct and comprises western North America (California and Nevada) and Macaronesia (Canary Islands, Tenerife). The connection of Macaronesia and America regarding their cryptogamic flora have been already described, but mainly referring to species present on the Azores and Madeira archipelagos, and implying disjunctions with tropical or Caribbean regions (Vanderpoorten *et al.*, 2007, 2011). Some of these species have their main distribution areas in America, as occurs here with *O. shevockii*. However, the disjunction reported here

between the Californian region and the Canary Islands is quite rare. Other spore-producing organisms, like lichens, show species with this type of distribution (Feuerer & Hawksworth, 2007), but among bryophytes, species that are present in both regions usually also expand their distribution into the Mediterranean basin (Shaw *et al.*, 2003; Werner *et al.*, 2003; Vigalondo *et al.*, 2016).

The dating analysis places the split of *Orthotrichum shevockii* from western North America and Tenerife at 0.44–6.69 Ma (Fig. 3.3.8). These dates are posterior to the origin of the Canary Islands (21 My) and match the time frame of the formation of Tenerife Island (3.5–11 My) (Carracedo *et al.*, 2007; Fernández-Palacios *et al.*, 2011). However, Las Cañadas area, where *O. shevockii* grows nowadays, dates back only 200 ka (Carracedo *et al.*, 2007). The phylogenetic inferences resolved *O. shevockii* within a clade composed of Californian endemic species, and the ancestral area estimation suggests a western North American origin for its ancestor (Figs. 3.3.7 and 8). According to these results, together with the volcanic origin and the age of the Macaronesian archipelagos and Tenerife Island, the present distribution of *O. shevockii* is likely to be the result of long-distance dispersal (LDD) from California to the Canary Islands. This confirms the hypothesis that recurrent events of LDD have occurred within *Orthotrichum* genus from western North America (California) to the Canary Islands (Patiño *et al.*, 2013). These events have taken place in different moments and reflect different dispersal windows, with the split of *O. underwoodii* and *O. handiense* being older than the disjunction of *O. shevockii* (Fig. 3.3.8).

The Californian origin of *Orthotrichum shevockii* supports the hypothesis of Vanderpoorten *et al.* (2011) that the Macaronesian cryptogamic flora is more related to the New World, at least for certain groups of bryophytes, whereas angiosperms are more connected to Europe and North Africa (Carine *et al.*, 2004). Moreover, it increases the evidence for bryophyte species with trans-Atlantic distributions, including Macaronesian taxa (Vanderpoorten *et al.*, 2007; Devos & Vanderpoorten, 2009; Patiño *et al.*, 2013b). In the case of the Canary Islands, trade winds that cross the Atlantic Ocean run from east to west –opposite to the direction that has been identified for this LDD– and they cannot explain this disjunction. On the contrary, the high altitude subtropical jet stream that does exactly the necessary route crossing over California and directly over the Canary Islands (Krishnamurti, 1961; Kuang *et al.*, 2014) seems to be a suitable vector for dispersal events

from west to east, as it has been suggested for other bryophyte species having a North America - Europe disjunction (Frahm, 2008).

The presence of *Orthotrichum shevockii* in the Canary Islands is restricted to a small area in Tenerife, while in western North America the species has a broader distribution including mountainous areas of California and nearest regions of Nevada. Restricted ranges in bryophytes are related to a recent origin, loss or lack of dispersal ability, preference for a specific habitat or a combination of some of these factors (Frahm, 2008). Our dating analyses do not discard a relative recent origin for the disjunction, which is placed between 0.44 and 6.69 Ma (Fig. 3.3.8). Concerning dispersal capabilities, all collected samples showed sporophytes with high numbers of spores that are small enough (12 µm in diameter) to be easily carried by wind through long distances (Gillespie *et al.,* 2012; Wilkinson *et al.,* 2012). Furthermore, it has been proved that Macaronesian bryophyte species do not necessarily lose their dispersal ability, maintaining connections between islands, archipelagos and near continents (Vanderpoorten *et al.,* 2008; Hutsemékers *et al.,* 2011; Laenen *et al.,* 2011; Patiño *et al.,* 2013a, 2015). Therefore, its restricted area is not a priori attributable to reproductive constraints, but likely to habitat limitations.

Most of the bryophyte species (endemic or not) that show restricted distributions in the Canary Islands grow in very rare habitats, especially in an altitudinal belt between 2000 and 3100 m a.s.l (González-Mancebo *et al.,* 2008; del Arco *et al.,* 2010). This matches the distribution of *Orthotrichum shevockii* in Tenerife, since it only appears growing on rocks in open arid zones at altitudes around 2100 m.a.s.l. On the Canary Islands archipelago, these altitudes are only also reached in La Palma Island, where the habitat favored by this moss is more restricted than in Tenerife. As for Azores and Cape Verde, the other Macaronesian archipelagos that exceed the elevation of 2000 m.a.s.l., this type of habitat is absent (Fernández-Palacios, 2011). Therefore, the distribution of *O. shevockii* in Macaronesia, which is restricted only to Tenerife, seems to be due to the lack of suitable habitats at high altitudes, or a combination of this with its closely recent origin, a hypothesis that needs to be checked by further studies of population genetics. Moreover, considering also the few known populations of *O. shevockii* in Tenerife, such studies would also help to infer the genetic diversity status of these populations for conservation purposes.

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Appendix

Selection of the samples of *Orthotrichum shevockii* used for morphological analyses. Numbers in bold between brackets preceding Herbarium codes correspond to the specimens included in molecular analyses as used in Figure 3.3.1 and Table 3.3.S1.

SPAIN: Canary Islands, Tenerife: Parque Nacional del Teide, cuadrícula 208, *J.M.B., J.G.M. & J.L.P s.n.*, 27 Mar 2008, TFCBry 15957; Parque Nacional del Teide, O del Valle de Chiñoque y al N de Montaña de La Cruz, *Losada-Lima s.n.*, 17 Oct 2008, TFCBry 16998; Parque Nacional del Teide, Barranco de La Zarza, base Montaña Los Asientos, *Losada-Lima s.n.*, 18 Apr 2008, TFCBry 15633; Parque Nacional del Teide, Cañada del Montón de Trigo, *Losada-Lima, León & Díaz s.n.*, 01 Jul 2008, TFCBry 15952; Parque Nacional del Teide, ladera del Teide, bajo Piedras Negras, *Losada-Lima s.n.*, 25 Apr 2008, **[15]** TFCBry 15858; Parque Nacional del Teide, Montaña Blanca, laderas

del Teide, *Losada-Lima, León & Díaz s.n.*, 02 Jul 2008, **[12]** TFCBry 15904, **[13]** TFCBry 15861, **[14]** TFCBry 15909; Parque Nacional del Teide, Montaña de los Pinos (de Arriba), *Losada-Lima s.n.*, 06 Mar 2009, **[16]** TFCBry 17428; Parque Nacional del Teide, Montaña de los Valles, *Losada-Lima s.n.*, 06 Mar 2009, TFCBry 17406; Parque Nacional del Teide, próximo a Diente del Risco, *Losada-Lima s.n.*, 11 Apr 2008, TFCBry 15567.

USA: California: Calaveras Co., Cave City Road, north of Dirty Gulch, *Norris 103406*, 20 Jan 2002, UC 1767898; Kern Co., off of the Pacific Crest Trail, south of Walker Pass to fork of Jack Creek, Kiavah Wilderness Scodie Mountains, *Shevock 13404 & D. York*, 11 May 1996, **[2]** CAS 958716 (paratype of *O. shevockii*); Lake Co., east of Round Mountain along Jerico Creek, *Toren 7061*, 26 Apr 1998, MAUAM 3288; Monterey Co., Los Padres Nat. Forest, *Shevock 29890*, 23 May 2007, CAS 1083201; Monterey Co., Los Padres Nat. Forest, Central Coast Ranges, Santa Lucia Range, Ventana Wilderness, south of Marble Peak, 23 May 2009, *Shevock 32935, Kellman & Lodder*, **[9]** MAUAM 5097, **[10]** NY 01140598; Mono Co., Benton Range, west of Benton Hot Springs, *Shevock 22289 & Glazer*, 28 May 2002, UC 1754201; Mono Co., Slimkard Creek, south of Topaz lake, *Shevock 21802 & Glazer*, 20 Feb 2002, **[6]** UC 1754431; Riverside Co., San Bernardino Nat. Forest, San Jacinto Mts., Bay Tree Spring, *Lara, Garilleti & Shevock s.n.*, 16 Nov 2008, **[1]** MAUAM 3291; San Bernardino Co., San Bernardino Nat. Forest, San Gorgonio Mts., Laurel Pines Camps, *Lara, Mazimpaka & Vigalondo s.n.*, 01 Jan 2013, MAUAM 3313; Santa Cruz Co., Big Basin Redwoods State Park, near Basin Trail and China Grade ~1.5 Mi beyond northern intersection W/ SR 236, 21 Jan 2001, *Kellman 1251* **[11]** CONN 00053520 (isotype of *O. kellmanii*); Shasta Co., Cassel-Fall River road, south of Fall Rivel Hills, *Norris 84716*, 3 Feb 1995, UC 1774443; Shasta Co., Cassel-Fall River road, south of Fall Rivel Hills*Norris 84724*, 3 Feb 1995, **[8]** UC 1774462; Tulare Co., trail to Crystal Cave in the vicinity of Cascade Trail, *Shevock 15768 & Tseng Yen-Hsuch*, 15 Jun 1997, **[4]** UC 1711731; Tulare Co., Sequoia Nat. Forest, Greenhorn Mountain, west of McNallys Fairview, *Shevock 16754 & S. Anderson*, 11 Dic 1997, **[3]** UC 1754230; Ventura Co., along Howard Ck., *Norris 55512*, 30 Dic 1979, UC 1649705; **Nevada:** Carson City Co., Carson Range, Voltaire Canyon, *Shevock 21948*, 05 Apr 2002, UC 1754323; Carson City Co., Carson Range, Voltaire Canyon, *Shevock 21948*, 05 Apr 2002, CAS 1040048; Carson City Co., Lake Tahoe Basin, Forest Service Vista Point, 39º09'48"N, 119º55'50"W, 6385 ft, *Shevock 22038*, 20 Apr 2002, CAS 1040116, **[7]** UC 1754264; Mineral Co., Toiyabe Nat. Forest, Anchorite Hills, near Anchorite Pass, *Lara, Garilleti, Shevock & Albertos s.n.*, 28 Oct 2008 , **[5]** MAUAM 3289, MAUAM 3290.

Supplementary Material

Table 3.3.S1. Specimens included in the molecular analyses. According to the guidelines, GenBank accession numbers will be provided after the manuscript is accepted. New accessions are in italic. Numbers between brackets after taxon ID correspond to the specimens included in molecular analyses as used in Figures 3.3.1 and 3.3.7. Samples originally identified as *Orthotrichum kellmanii* appear under this name in the table.

				Genbank accession number			
Taxon	**ID**	**Locality**	**Voucher**	**ITS2**	***rps*4**	***trn*L-F**	***atp*B-*rbc*L**
Macrocoma lycopodioides	BV024	South Africa, Western Cape	MAUAM 2953	KT862258	KT862288	*XX000000*	*XX000000*
Nyholmiella obtusifolia	O118	Spain, Burgos	MAUAM 4343	--	JQ836797	JQ836986	JQ836695
N. obtusifolia	O119	France, Haute-Savoie	MAUAM 4342	--	JQ836798	JQ836987	JQ836696
Lewinskya acuminata	BV006	France, Corse	MAUAM 3164	KT862262	KT862292	*XX000000*	*XX000000*
L. acuminata	BV010	Spain, Ávila	MAUAM 3272	KT862263	KT862293	*XX000000*	*XX000000*
L.affinis	BV015	Spain, Jaén	MAUAM 4448	KT862277	KT862306	*XX000000*	*XX000000*
L.fastigiata	BV016	Turkey, Artvin	MAUAM 4449	KT862278	KT862307	*XX000000*	*XX000000*
Orthotrichum alpestre	R669	Turkey, Gümüshane	MAUAM 4391	*XX000000*	JQ836864	JQ837053	JQ836760
O. alpestre	R670	Switzerland, St. Gallen	MAUAM 1685	*XX000000*	JQ836865	JQ837151	JQ836761
O. anomalum	O120	Spain, Asturias	MAUAM 4330	*XX000000*	JQ836799	JQ836988	JQ836697
O. bartrami	R598	USA, Arizona	CAS sn	*XX000000*	JQ836838	JQ837027	JQ836734
O. bistratosum	BV088	Spain, Jaén	MAUAM 4594	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. casasianum	R398	Spain, Álava	MAUAM 1702	*XX000000*	JQ836811	JQ837000	JQ836707
O. columbicum	R674	Canada, British Columbia	MAUAM 4284	*XX000000*	JQ836874	JQ837063	JQ836770
O. columbicum	R678	Spain, León	MAUAM 657	*XX000000*	JQ836877	JQ837066	JQ836773
O. comosum	R655	Spain, Almería	MAUAM 4359	*XX000000*	JQ836852	JQ837041	JQ836748
O. comosum	R673	Spain, Cadiz	MAUAM 4361	*XX000000*	JQ836860	JQ837049	JQ836756
O. confusum	R680	USA, California	MAUAM 4323	*XX000000*	JQ836878	JQ837067	JQ836774
O. consimile	R677	USA, California	MAUAM 4278	*XX000000*	JQ836869	JQ837058	JQ836765
O. consimile	R616	USA, California	UC 1760062	*XX000000*	JQ836870	JQ837059	JQ836766
O. coulteri	R564	USA, California	MAUAM 4367	*XX000000*	JQ836817	JQ837006	JQ836713
O. coulteri	R562	USA, California	MAUAM 4368	*XX000000*	JQ836815	JQ837004	JQ836711
O. cuculatum	R577	USA, California	MAUAM 4381	*XX000000*	JQ836830	JQ837019	JQ836726
O. cuculatum	R579	USA, California	MAUAM 4380	*XX000000*	JQ836829	JQ837018	JQ836725
O. flowersii	R632	USA, Nevada	CAS-1045756	--	JQ836842	JQ837031	JQ836738
O. franciscanum	R570	USA, California	MAUAM 4390	*XX000000*	JQ836823	JQ837012	JQ836719
O. franciscanum	R571	USA, California	UC 1739290	*XX000000*	JQ836824	JQ837013	JQ836720
O. hallii	BV089	USA, Nevada	MAUAM 4595	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. halii	BV090	USA, Nevada	MAUAM 4596	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. handiense	O2115	Spain, Canary Is., Fuerteventura	MAUAM 4689	*XX000000*	JX297214	JX297224	JX297209
O. handiense	O2172	Spain, Canary Is., Fuerteventura	MAUAM 4690	*XX000000*	JX297215	JX297225	JX297210
O. handiense	O2173	Spain, Canary Is., Fuerteventura	MAUAM 2043	*XX000000*	JX297216	JX297226	JX297211
O. kellmanii	BV054 [9]	USA, California	MAUAM 5097	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. kellmanii	BV103 [10]	USA, California	NY 01140598	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. kellmanii	1502 [11]	USA, California	CONN00053520	--	*XX000000*	*XX000000*	*XX000000*
O. norrisii	R567	USA, California	MAUAM 4395	*XX000000*	JQ836820	JQ837009	JQ836716
O. norrisii	R568	USA, California	UC 1741966	*XX000000*	JQ836821	JQ837010	JQ836717
O. persimile	R580	USA, California	UC 1650645	*XX000000*	JQ836833	JQ837022	JQ836729
O. persimile	R666	USA, California	MAUAM 4327	*XX000000*	JQ836857	JQ837046	JQ836753
O. pilosisimum	R640	USA, Nevada	MAUAM 4334	*XX000000*	JQ836845	JQ837034	JQ836741
O. pilosisimum	R644	USA, Nevada	MAUAM 4333	*XX000000*	JQ836847	JQ837036	JQ836743
O. pulchellum	R682	Canada, British Columbia	MAUAM 4336	*XX000000*	JQ836880	JQ837069	JQ836776
O. pulchellum	R684	Spain, Álava	MAUAM 4338	*XX000000*	JQ836882	JQ837071	JQ836778
O. pumilum	R152	Estonia	TU 170	--	JQ836802	JQ836991	JQ836700
O. scanicum	R018	Greece, Sterea Hellada	MAUAM 2166	*XX000000*	JQ836800	JQ836989	JQ836698
O. schimperi	R364	Tunisia, Aïn-Draham	MAUAM 2448	*XX000000*	JQ836810	JQ836999	JQ836706
O. schimperi	R656	USA, California	MAUAM 4339	*XX000000*	JQ836853	JQ837042	JQ836749

Table 3.3.S1. Continuation**.**

				Genbank accession number			
Taxon	**ID**	**Locality**	**Voucher**	**ITS2**	***rps*4**	***trn*L-F**	***atp*B-*rbc*L**
O. sharpii	R769	Mexico, Veracruz	MAUAM 4340	--	JQ837050	JQ836861	JQ836757
O. shevockii	BV030 [5]	USA, Nevada	MAUAM 3289	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. shevockii	BV031 [2]	USA, California	CAS 958716	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. shevockii	BV032 [8]	USA, California	UC 1774462	*XX000000*	*XX000000*	*XX000000*	--
O. shevockii	BV043 [4]	USA, California	UC 1711731	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. shevockii	BV044 [6]	USA, California	UC 1754431	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. shevockii	BV045 [3]	USA, California	UC 1754230	*XX000000*	*XX000000*	*XX000000*	--
O. shevockii	BV046 [7]	USA, Nevada	UC 1754264	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. shevockii	BV048 [1]	USA, California	MAUAM 3291	*XX000000*	*XX000000*	--	*XX000000*
O. shevockii	BV049 [12]	Spain, Canary Islands, Tenerife	TFCBry 15904	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. shevockii	BV050 [13]	Spain, Canary Islands, Tenerife	TFCBry 15861	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. shevockii	BV051 [16]	Spain, Canary Islands, Tenerife	TFCBry 17428	*XX000000*	*XX000000*	--	*XX000000*
O. shevockii	BV052 [14]	Spain, Canary Islands, Tenerife	TFCBry 15909	*XX000000*	*XX000000*	*XX000000*	--
O. shevockii	BV053 [15]	Spain, Canary Islands, Tenerife	TFCBry 15858	*XX000000*	*XX000000*	*XX000000*	*XX000000*
O. tenellum	R294	Italy, Sicily	MAUAM 4346	*XX000000*	JQ836805	JQ836994	JQ836703
O. tenellum	R295	Portugal, Tras os Montes e Alto Dour	MAUAM 4347	*XX000000*	JQ836806	JQ836995	JQ836704
O. underwoodii	R583	USA, California	MAUAM 4341	*XX000000*	JQ836835	JQ837024	JQ836731
Zigodon pentastichus	ID207	Argentina, Córdoba	MAUAM 2981	KT862260	KT862290	*XX000000*	*XX000000*
Z. viridissimus	ID208	United Kingdom	MAUAM 2910	KT862259	KT862289	*XX000000*	*XX000000*

Table 3.3.S2. Marginal likelihood (MLE) and Bayes factor (BF) values for alternative clocks and models tested in BEAST. The best model is marked in bold.

		Path Sampling		**Stepping-Stone**	
		ln (MLE)	2ln (BF)	ln (MLE)	2ln (BF)
Uncorrelated log-normal	**Birth-death**	**-5781,254**	**0,000**	**-5774,789**	**0,000**
	Yule	-5799,834	37,161	-5801,716	40,924
Strict consensus	Birth-death	-5819,606	76,704	-5820,374	78,239
	Yule	-5826,930	91,352	-5828,690	94,871

Figure 3.3.S1. Results of the principal component analysis (PCA) representing the first three components. The percentage of variance explained by each component is given between brackets. Arrows represent the variables included in the analyses. cos2 represent the squared loadings for variables. ! = samples originally identified as *O. kellmanii*.

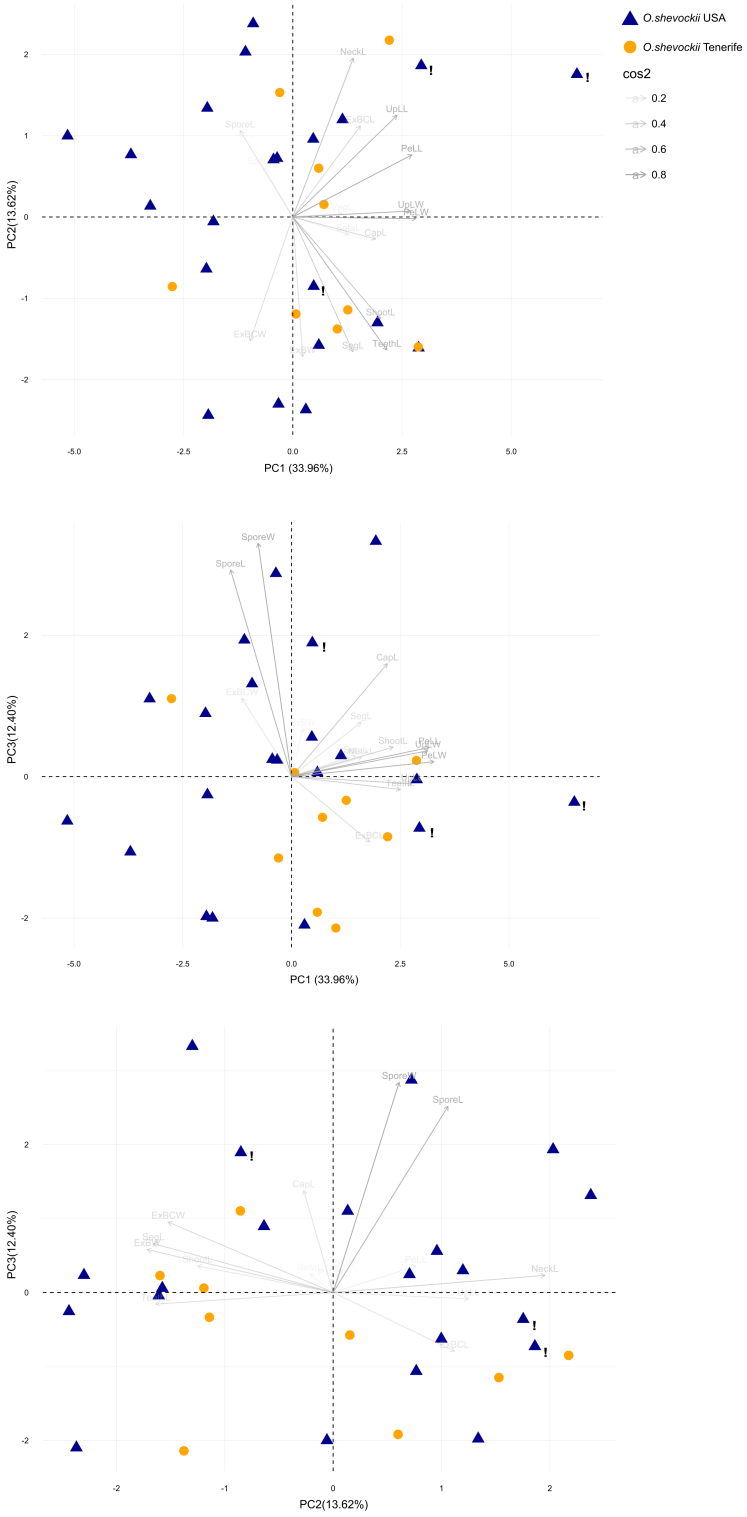
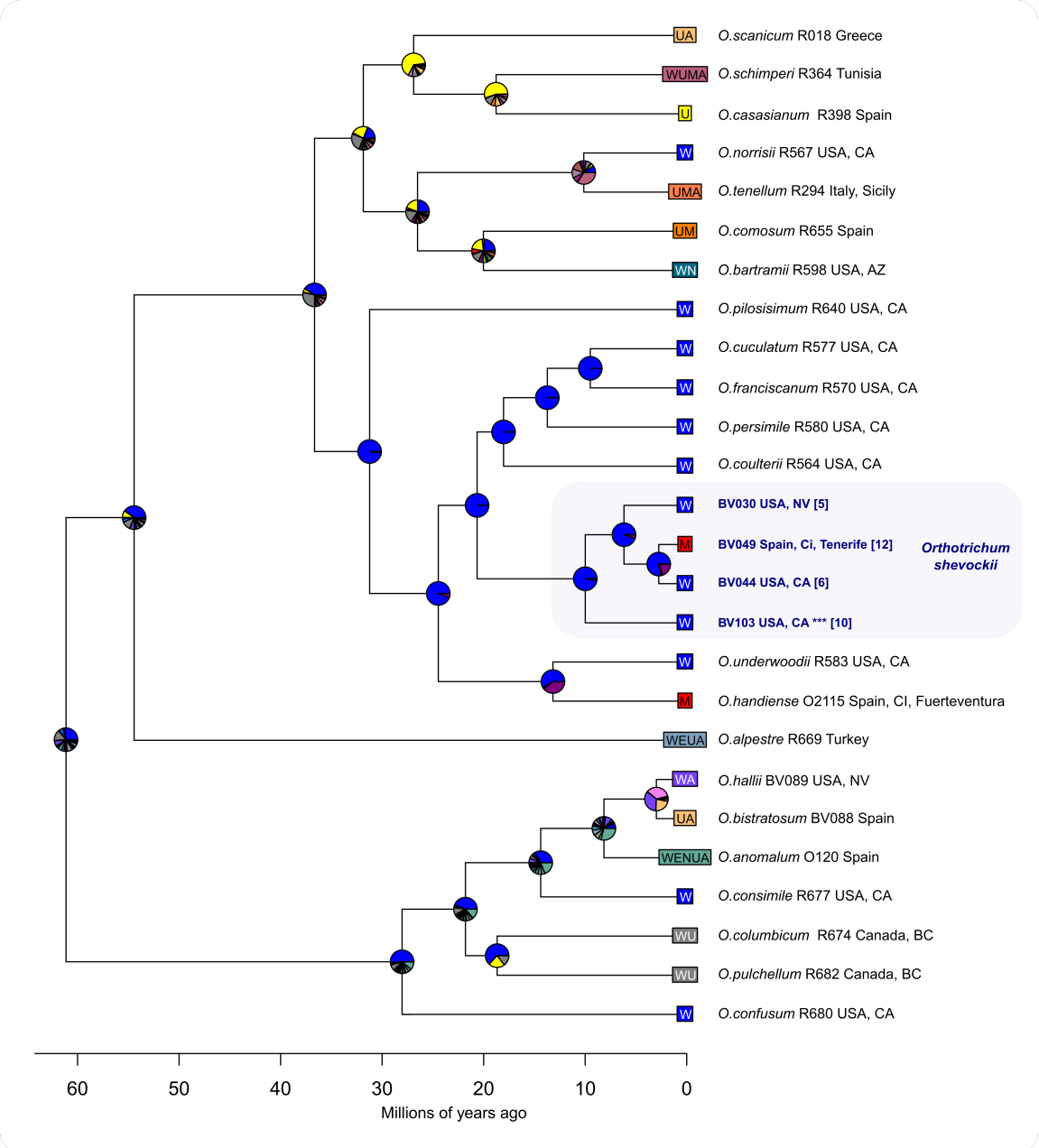


Figure 3.3.S2. Chronogram of the phylogenetic relationships among the four loci and ancestral area estimations for *Orthotrichum shevockii* and the evaluated ingroup under the DEC + J model implemented in BioGeoBEARS (ancestral states: global optim, 6 areas max. d=0.0059; e=0; j=0.0206; LnL=−72.55). The probabilities of each biogeographical region are presented. Letters in colour boxes correspond to the following ancestral areas or combination of areas: W = western North America; E = Eastern North America; N = Neotropics; M = Macaronesia; U = Europe; A = Asia.



Chapter

3.4

Lewinskya affinis

Do mosses really exhibit larger distribution ranges than angiosperms? Insights from the study of the *Lewinskya affinis* complex.

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Pending of publication

Abstract

The strikingly lower number of bryophyte species, and in particular of endemic species, in comparison with angiosperms has traditionally been interpreted in terms of their low diversification rates associated with a high long-distance dispersal capacity. Such feature would have hampered the chances for allopatric speciation, resulting in large transoceanic distribution ranges. This hypothesis is tested here with *Lewinskya affinis* (Schrad. ex Brid.) F.Lara, Garilleti & Goffinet (≡ *Orthotrichum affine* Schrad. ex Brid.), a moss widely spread across Europe, North Africa, East Africa, southwestern Asia, and western North America. We implemented an integrative species delimitation approach and tested different competing taxonomic hypotheses derived from the analysis of morphological and multilocus sequence data. The best hypothesis selected by a Bayes factor delimitation analysis applied to the molecular dataset, involved that *L. affinis s.l.* is actually a complex of no less than seven distinct species, resolved in a monophyletic clade that also includes the two already known and well-defined *L. tortidontia* and *L. praemorsa*, namely *L. affinis* complex. Discriminant analyses looking for the best combination of morphological traits separating the seven species within *L. affinis s.l.* indicate that each can be identified morphologically with a minimal error rate. These species are *L. affinis s.str.*, two resurrected synonyms *L. fastigiata* and *L. leptocarpa*, and 4 new species *L. scissa* sp. nov., *L. arida* sp. nov., *L. pacifica* sp. nov. and *L. pseudoaffinis* sp. nov. Each of these new or re-circumscribed species is restricted to an area that ranges from an archipelago like the Canary Islands to a biogeographic region such as East Africa or western North America, suggesting an underestimation of the number of moss species, and an overestimation of the species distribution ranges. Our findings clearly call for a re-evaluation of the plethora of the so-called 'cryptic' bryophyte species that has been discovered with the advance of molecular techniques and species delimitation analyses during the past 20 years.

Introduction

Bryophytes represent a taxonomically challenging group due to their reduced morphologies as compared to other embryophytes. Although they are the second most diverse group of terrestrial plants, the considerable lower number of species in the group and, in particular, of endemic species in comparison with angiosperms (Vanderpoorten *et al.,* 2010), has traditionally been interpreted in terms of the high dispersal capacities of bryophytes and their consequently large distribution ranges (Medina N.G. *et al.,* 2011). This feature implicitly suggests that bryophytes require large geographic areas to speciate in allopatry (Kisel & Barraclough, 2010). Mounting evidence for geographic structuring of bryophyte phylogeographies (Désamoré *et al.,* 2016; Kyrkjeeide *et al.,* 2016), however, raises the question of whether patterns of species richness and distribution in traditionally defined bryophyte species truly characterize the lack of intercontinental geographic barriers or rather reflect taxonomical shortcomings. In fact, an increasing number of studies reported that apparently widely distributed bryophyte species correspond to complexes of multiple taxa, not necessarily sister, with much narrower distribution ranges (e.g. Heinrichs *et al.,* 2010; Stech *et al.,* 2013; Lang *et al.,* 2015; Carter *et al.,* 2017). However, still few studies perform a subsequent morphological re-evaluation of the species discovered on molecular phylogenetic bases or follow integrative approaches (e.g. Medina R. *et al.,* 2012, 2013; Renner *et al.,* 2013; Aranda *et al.,* 2014; Hedenäs *et al.,* 2014; Draper *et al.,* 2015; Caparrós *et al.,* 2016; Patiño *et al.,* 2017, Sim-Sim *et al.,* 2017). Thereby, this implies that there is still a considerable bias between number of species discovered and those that are formally described (Pante *et al.,* 2014).

Lewinskya affinis (Schrad. ex Brid.) F.Lara, Garilleti & Goffinet (≡*Orthotrichum affine* Schrad. ex Brid.) is considered one of the most common Orthotrichaceae species in Europe and North Africa and an important element of the epiphytic flora of these regions, but also

is acknowledged to occur in western North America, southwestern Asia, and East Africa. Several authors have considered that *L. affinis* exhibits a substantial morphological variation across its distribution range (Grout, 1935; Nyholm, 1956; Vitt, 1973; Lewinsky, 1978, 1998; Smith, 2004; Lara & Garilleti, 2014), which has historically led to contrasting taxonomic treatments (Lewinsky, 1978; Plášek *et al.,* 2011; Lara & Garilleti, 2014; for a review see Frahm, 2011). A few studies have further noted its resemblance and possible confusion with other species like *L. speciosa* (Nees) F.Lara Garilleti & Goffinet (Grout, 1935; Nyholm, 1956; Frahm, 2011; Lara & Garilleti, 2014) or *L. tortidontia* (F.Lara, Garilleti & Mazimpaka) F.Lara, Garilleti & Goffinet (Lara & Garilleti, 2014). Recently, Frahm (2011) and Lara & Garilleti (2014) pointed out differences in several morphological characters along European specimens of *L. affinis* that strongly suggested the existence of two separate taxa, although no formal recognition was proposed. Furthermore, in materials ascribed to *L. affinis* from different collection campaigns trough western North America, Canary Islands and East Africa, we noticed some significant morphological differences across the geographic areas. This could be pointing out to the existence of several morphotypes within *L. affinis,* of no less than two different ones for North America, one for East Africa and the two suggested for Europe and North Africa by Frahm (2011) and Lara & Garilleti (2014).

In this framework, we address here the following questions: 1) Does *Lewinskya affinis* correspond to a single species as it has been thought so far, or is there more than one taxon included within it? In this latter case, 2) to what extent can these taxa be morphologically and molecularly recognized? 3) Is the morphological similarity of these taxa due to morphological convergence or are they sibling species? 4) Do these taxa exhibit allopatric or sympatric distribution ranges?

To answer these questions, we implemented an integrative species delimitation analysis testing competing hypotheses derived from our preliminary observations, previous taxonomic circumscriptions of *Lewinskya affinis,* and those resulting from complementary analyses of multilocus DNA sequence and morphological datasets in this study. Molecular methods include phylogenetic inferences, GYMC species delimitation and Bayes Factor delimitation (BFD) analyses, while morphological analyses are addressed by multivariate statistical analyses such as Principal components (PCA) and Discriminant function (DFA) analyses. The congruence of all analyses is tested as well as the significance of the best species hypothesis in order to identify and describe the possible species configuring the *L. affinis* complex.

Material and methods

The integrative methodological approach of this work involves different aspects that are presented independently below, although they are interrelated as shown in Figure 3.4.1.

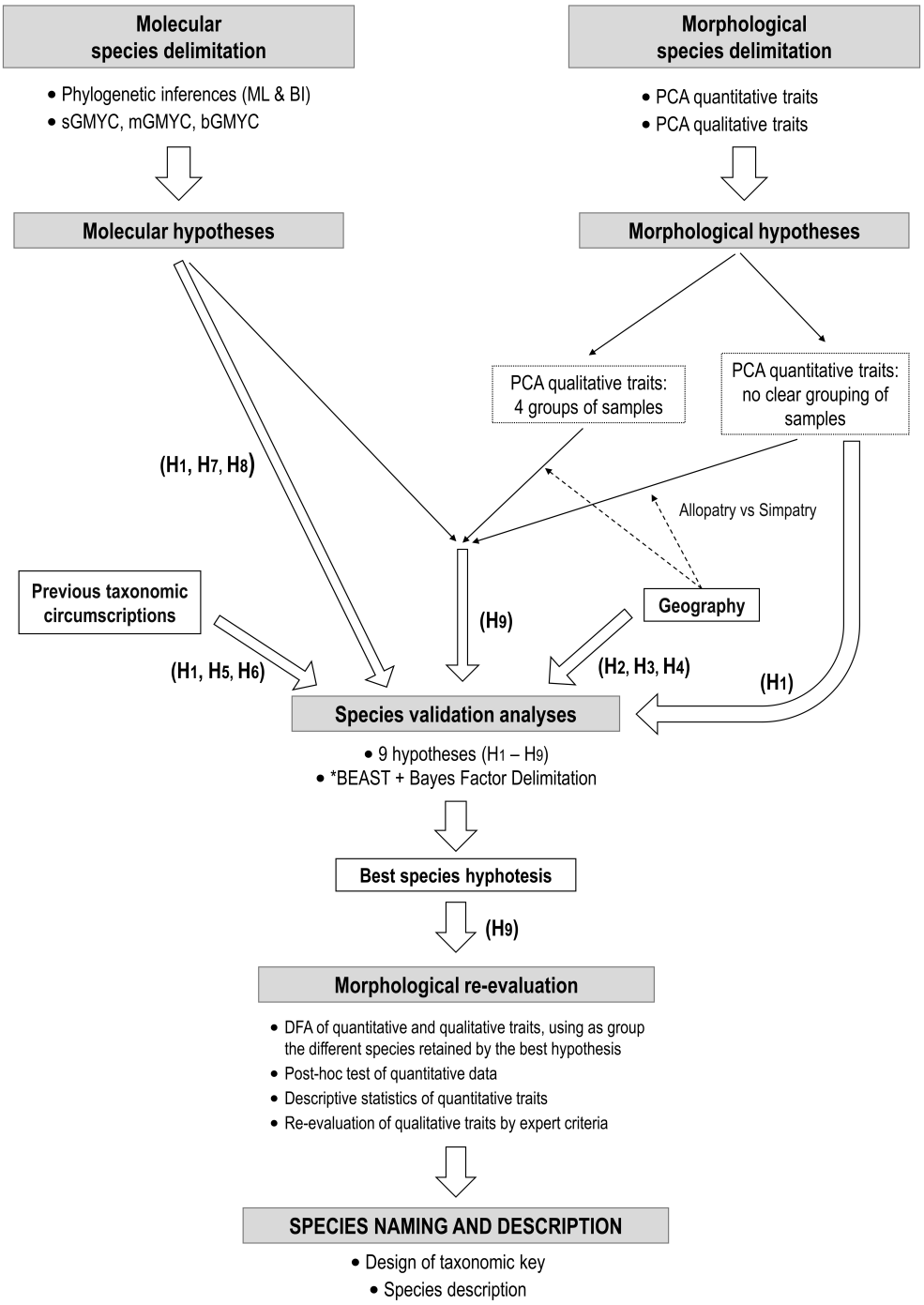

Figure 3.4.1. Outline of the methodological procedure performed for species identification and delimitation in the *Lewinskya affinis* complex. See Material and Methods section for detailed information of each step. H1-H9 refer to the hypotheses included in Table 3.4.2.

Taxon sampling and molecular protocols

Ninety specimens were selected in order to cover the distribution range of *Lewinskya affinis*, including 39 from Europe, 4 from Macaronesia, 3 from North Africa, 13 from East Africa, 2 from southwestern Asia, and 29 from western North America (Fig. 3.4.2 B-C). For molecular analyses, only 70 of the 90 specimens could be amplified. Forty-one sequences of other *Lewinskya* species from all continents were included to provide a phylogenetic framework to investigate whether *L. affinis* is a monophyletic lineage. Four species from the genera *Macrocoma* (Hornsch. ex Müll.Hal.) Grout, *Pulvigera* Plášek, Sawicki & Ochyra, and *Zygodon* Hook. & Tayl. were included as outgroups. Voucher information and Genbank accession numbers are listed in the Appendix.

DNA was extracted from apices of stems and branches of dried specimens using mainly the DNeasy® Plant Mini Kit (Qiagen, Valencia, California, USA), but also the standard CTAB protocol (Doyle & Doyle, 1987). Nucleotide sequences were obtained from four genomic regions: two from the chloroplast genome (*rps*4, *rpl*32-*trn*L$^{(UAG)}$), and two nuclear expressed sequence tag (EST) from McDaniel *et al.* (2013): AW086770–115 and AW098158–317 (hereafter termed EST-115 and EST-317 respectively, see Table 3.4.1). The PCRs were performed using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech Inc) in a final reaction volume of 25 µl according to the manufacturer's instructions, with 2-4 µl of template DNA for rps4 and rpl32, and 5-10 µl of template DNA for both ETS regions. The amplification for *rps*4 consisted of 5 min at 94°C, followed by 30 cycles of 30 at 95°C, 1 min at 52°C and 30 s at 68°C with a final extension step of 7 min at 68ºC. For *rpl*32-*trn*L$^{(UAG)}$, the protocol was 5 min at 80ºC with 30 cycles of 1 min at 95ºC, 1 min at 50ºC, followed by a ramp of 0.3ºC/s to 65ºC, 4 min at 65ºC and a primer extension of 5 min at 65ºC. The cycling conditions for both ETS regions were 4 min at 94ºC then 10 cycles of 30 s at 94°C, 45 s with an annealing temperature of 62°C that decreased one degree each cycle, and 45 s at 72ºc, followed by 25 cycles of 30 s at 94°C, 45s s at 52°C for 30 s and 45 s at 72°C, with a final extension step of 4 min at 72ºc. PCR products were purified using Exo/SAP protocol (Thermo Fisher Scientific, Spain). Samples were incubated with 1 µl of Exo1 enzyme and 4 µL of FastAP following the manufacturer's instructions. Cleaned PCR products were sequenced by Macrogen (www.macrogen.com).

For each DNA region, forward (5'–3') and reverse (3'–5') sequences were edited and assembled into contigs using Geneious 9.0.2 (http://www.geneious.com; Kearse *et al.*, 2012). Sequences were trimmed at both ends and aligned using the plugin MUSCLE (Edgar,

2004) in Geneious, and then edited manually, inserting gaps where necessary to preserve positional homology.

Table 3.4.1. Sequences of primers used for PCR amplification and sequencing.

Region	Primer name and sequence (5'–3')	
*rps*4	*rpsA*	ATGTCCCGTTATCGAGGACCT
	trnaS	TACCGAGGGTTCGAATC
*rpl*32-*trn*L$^{(UAG)}$	*rpL32-F*	CAGTTCCAAAAAAACGTACTTC
	trnL$^{(UAG)}$	CTGCTTCCTAAGAGCAGCGT
ETS-115	*ETS-115F*	TCCGCGAGCTCTGAGTGG
	ETS-115R	AACAACTTCACCACATCTGCACG
ETS-317	*ETS-317F*	CGGGCTTGGTCTGTCCTCC
	ETS-317R	TCTTCTGCCCTGGGAAGGC

Phylogenetic analyses

Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML). The best-fitting substitution models for each locus were inferred under the Bayesian Information Criterion (BIC) in jModelTest v.2.1.3 (Darriba *et al.,* 2012). Maximum likelihood analyses were run with RAxML 8 (Stamatakis, 2014), and the best ML tree was selected from 100 iterations and its support was assessed with 1000 replicates of bootstrap resampling under the ML criterion. Bayesian inference phylogenetic analyses were carried out using MrBayes v.3.2.1 (Ronquist *et al.,* 2012) at CIPRES Science Gateway (www.phylo.org; Miller *et al.,* 2010). The Markov chain Monte Carlo (MCMC) simulation was run for 2 millions of generations for each independent loci and 5 millions for the multilocus dataset, with two runs and four chains, sampling trees and parameters every 1000 generations. After checking that stationarity had been reached (i.e. the average standard deviation of split frequencies remained below 0.01 for the last 10,000 generations), posterior probabilities (PP) were estimated from the 50% majority-rule consensus trees after a burn-in of 25% of the starting trees. The resulting trees for both ML and BI were plotted using FigTree v.1.4.2 (Rambout, 2012).

The four loci independent data sets were combined in a single concatenated matrix, after checking visually the congruence of independent analyses for each locus in branches with high support (PP $\geq$ 0.95 and BS $\geq$ 85). The concatenated data set was analysed in PartitionFinder (Lanfear *et al.,* 2012) to select the best partitioning scheme, using the greedy

algorithm with linked branch lengths under the BIC. For each of the three partitions defined (rpl32-rps4, EST-317 and EST-115) the best-fit substitution model was a HKY+G model.

In order to produce ultrametric trees necessary for species delimitation analyses (see below), we ran strict clock and uncorrelated lognormal relaxed clock analyses of the combined molecular dataset using BEAST v.1.8 (Drummond *et al.*, 2012). Because the inclusion of identical sequences results in many zero-length branches at the tip of the tree and can cause the model to overpartition the dataset (Reid & Carstens, 2012), we reduced our list of specimens to haplotypes (resulting in 33 sequences) using DNAsp v. 5. (Librado & Rozas, 2009) and rechecked visually in PhyDE (Müller *et al.*, 2006). We ran BEAST analyses with the same best partitions scheme and substitutions models described above for the multilocus dataset. Four chains were run for 100 million generations and sampled every 104 generations under a birth-death and a Yule speciation model, respectively. Convergence and mixing of the four chains was assessed by checking that all parameters had reached stationarity and sufficient (> 200) effective sample sizes using Tracer v.1.6 (Rambaut *et al.*, 2014), and 1,000 trees were discarded as burn-in. Based on Marginal likelihood estimates (MLEs) and Bayes factors the uncorrelated lognormal relaxed clock under a birth-death model was selected (Table 3.4.S1) and employed in subsequent analyses.

Molecular species delimitation analyses

We used the General Mixed Yule-Coalescent (GMYC) model to define preliminary species delimitation hypotheses. The GMYC model attempts to distinguish between interspecific (modeled by a Yule process) and intraspecific (modeled by the coalescent one) branching events on a phylogenetic tree, based on the idea that the rate of coalescence should be much higher within than between species. Two first implementations of the GMYC model are based on a likelihood approach that combines such equations from coalescent and Yule models to define a single (sGYMC, Pons *et al.*, 2006) or multiple thresholds (mGYMC, Fujisawa & Barraclough, 2013) representing the species boundaries on ultrametric gene trees. These analyses were performed on the maximum clade credibility tree of the BEAST analyses (see above), without removing the phylogenetically closer taxa (Powell, 2012), using the SPLITS package (Ezdar *et al.*, 2009). The best-fit GMYC model was determined using a likelihood ratio test. We also used a Bayesian version of the GMYC model (bGMYC, Reid & Carstens, 2012) that accounts for error in phylogeny estimation and uncertainty in model parameters (Monaghan *et al.*, 2009). We randomly sampled 100 trees from the

posterior distribution of the BEAST analyses and ran the GMYC analyses on each tree for 50,000 generations, discarding the first 10,000 generations as burn-in, and using a thinning interval of 100 (as recommended by Monaghan *et al.*, 2009). These analyses were performed using the bGMYC package (Monaghan *et al.,* 2009) available from "http://R-Forge.R-project.org" for R (R Core Team, 2016).

Morphological species delimitation analyses

Multivariate statistical morphometric analyses were computed to define a morphological hypothesis. Twenty-one quantitative and 25 qualitative characters were selected for their relevance for species circumscriptions within Orthotricheae (see chapter 3.1, Vigalondo *et al.*, 2016) or for exhibiting variation within *Lewinskya affinis* according to different authors (for review see Frahm, 2011; Lara & Garilleti, 2014). Qualitative characters included multistate and binary characters (Table 3.4.S2). For the purpose of multivariate analyses (see below), qualitative multistate characters were transformed into additive binary traits, resulting in a total of 52 variables. For morphological analyses, 81 of the 90 specimens were included, being the exclusion of the remaining nine due to the bad conditions of sporophytes for several characters. For plant size, the length was measured on five individual shoots (specimens) from each collection. One of these specimens was selected at random and five replicates were taken for each of the remaining quantitative and qualitative characters to consider the within-plant phenotypic variation. For sporophytic traits, measurements were performed on 1–5 capsules depending on their availability for each specimen. Means from replicates for each character were then calculated to construct the final data set employed in statistical analyses.

Principal Component Analysis (PCA) were implemented to explore whether correlated suites of traits allowed to identify distinct morphotypes. This analysis was performed independently on each of the quantitative and qualitative data sets, on a correlation matrix with morphological variables scaled, due to the heterogeneous nature of the variables scored. These multivariate analyses were run twice: (i) discarding samples with missing values; and (ii) also replacing missing values by the mean value of each character. Results from both approaches were congruent (results not shown), so to avoid reducing our sampling size, we used the data set with missing values replaced by the mean for the final analyses. Statistical analyses were implemented using R v.3.3.1 (R Core Team, 2016).

Table 3.4.2. Alternative species delimitation hypotheses tested using validation approaches through Marginal likelihood (MLE) and Bayes factor (BFD) and the resulted values for each of them. The number of species (sp) considered in each hypothesis follows the hypothesis number.

			Path Sampling (PS)		Stepping-Stone (SS)	
Hypothesis	**Distinct species**	**Motivation**	ln (MLE)	2ln (BF)	ln (MLE)	2ln (BF)
H_1 (1 sp)	One widespread species (*L. affinis*).	Morphological similarity and previous circumscriptions	-4852,74	138,33	-4854,10	138,72
H_2 (2 sp)	One Holarctic species (*L. affinis*) and one from East Africa (*L. leptocarpa*).	Geographic isolation I	-4850,88	134,61	-4852,39	135,29
H_3 (2 sp)	One species in the Old World (*L. affinis*) and one in the New World (*L. sp.*).	Geographic isolation II	-4834,45	101,74	-4834,64	99,79
H_4 (3 sp)	One species in each of the three disjunct areas: western Palearctic (*L. affinis*), East Africa (*L. leptocarpa*), and western North America (*L. sp*).	Geographic isolation III	-4828,96	90,78	-4830,37	91,25
H_5 (3 sp)	One widespread species in the world (*L. affinis*), one in western North America (*L. praemorsa*), and one in Europe (*L. tortidontia*).	Accepted taxonomical situation since the world revision of Lewinsky (1993)	-4847,97	128,80	-4847,94	126,39
H_6 (6 sp)	One Holarctic species (*L. affinis*), one in East Africa (*L. leptocarpa*), one in western North America (*L. praemorsa*), and other three taxa in Europe (*Orthotrichum affine* var. *bohemicum*, *L. fastigiata*, and *L. tortidontia*).	All taxa related to *L. affinis* once considered by literature	-4829,67	92,20	-4831,14	92,79
H_7 (7 sp)	One species in western Palearctic and East Africa (*L. affinis*), plus two in Europe and the Mediterranean (*L. fastigiata, L. tortidontia*); four in western North America (*L. praemorsa, L. arida, L. pacifica, L. pseudoaffinis*).	Phylogeny I	-4790,18	13,21	-4791,35	13,21
H_8 (8 sp)	One species in western Palearctic and East Africa (*L. affinis*), plus one in Canary Islands (*L. scissa*), and two in Europe and the Mediterranean (*L. fastigiata, L. tortidontia*); four in western North America (*L. praemorsa, L. arida, L. pacifica, L. pseudoaffinis*).	Phylogeny II , GMYC	-4790,10	13,04	-4791,27	13,05
H_9 (9 sp)	Three species in Europe and North Africa (*L. affinis, L. fastigiata, L. tortidontia*); one in Canary Islands (*L. scissa*); one in East Africa (*L. leptocarpa*); four in western North America (*L. praemorsa, L. arida, L. pacifica, L. pseudoaffinis*).	Morphology, phylogeny and geography	**-4783,57**	0.00	**-4784,74**	0,00

Species hypothesis validation

Nine different species hypotheses were formulated including those resulting from the molecular (ML, BI and GMYC tree topologies) and morphological (PCA) analyses together with other based on previous taxonomic circumscriptions of *Lewinskya affinis* (e.g. Nyholm, 1956; Lewinsky, 1978, 1998; Plášek *et al.*, 2011) and different geographic hypotheses (Table 3.4.2). These hypotheses were tested using a Bayes factor delimitation (BFD; see Grummer *et al.*, 2014). This approach uses a Bayesian coalescent-based reconstruction of species trees for a range of delimitation models that involve the lumping and splitting of hypothesized species, and compares their marginal likelihoods using Bayes factors (Grummer *et al.*, 2014).

For each of the nine competing species hypotheses we performed a Bayesian reconstruction of the species tree using *BEAST v1.8.0 (Drummond *et al.*, 2012) with the same reduced matrix that was used for the GMYC analyses, and the best partitions scheme and substitutions models described above. For *BEAST specifications, we followed Hotaling *et al.* (2016). Gene trees were estimated using a relaxed uncorrelated lognormal clock. For all partitions, a uniform prior was used for the uncorrelated lognormal clock with an initial value of 1.0 and an upper bound of 25. The species tree was estimated using a Birth-Death prior with a piecewise linear and constant root population-size model. An inverse gamma distribution was used for the population mean prior with an initial value of 0.02, shape set at 3.0, and scale set at 0.3. An inverse gamma distribution was also used for the Birth-Death mean growth rate, with an initial value of 1.0, shape set at 0.5, and scale set at 1.0. For each tested hypothesis two *BEAST replicates were run independently for 100 million generations, and sampled for trees and parameters every 20,000 generations. The first 10% of the trees of each run was discarded as burn-in after visual inspection of chain stationarity and convergence with Tracer v.1.6 (Rambaut *et al.*, 2014).

Marginal likelihoods estimates (MLEs) measured as log likelihoods, were then calculated from the Bayesian posterior distributions using path-sampling (PS, Lartillot *et al.*, 2006) and stepping-stone (SS, Xie *et al.*, 2011) methods, with 100 path steps, a chain length of 100,000 generations and likelihoods saved every 1,000 generations. The resulted MLEs were averaged across replicate runs to generate a single PS and SS value for each hypothesis. The obtained MLEs for all hypotheses were ranked, and Bayes factors were then calculated

as two times the difference between the best-fitting model MLE (-lnHypA) and each alternative model MLEs (-lnHypB) [i.e. 2*(-lnHypA – -lnHypB)]. Values of 2lnBf in the range of 0–2 are generally interpreted as indicating no difference in support for two models, while a 2lnBf > 10 indicates 'decisive' support in favour of the best-fitting model over its alternative hypothesis (Kass & Raftery, 1995). Grummer *et al.* (2014) recognized distinct lineages with a 2lnBf > 10 and we here follow these guidelines.

Morphological species re-evaluation

To test whether the best species hypothesis retained by the BFD is morphologically relevant, a final morphological re-evaluation of all specimens was performed at qualitative and quantitative level, to also establish the best morphological trait combination that allows the characterization of each of the new delimited species. First discriminant function analysis (DFA) was implemented, including as groups the species recovered by the best hypothesis of BFD analysis. These analyses are sensitive to multicollinearity and are designed to work with matrices including more observations in the category with the lowest sampling size than variables. To reduce the number of variables and solve the problem of multicollinearity, we employed the three first morphological PCA axes of quantitative and qualitative analyses as new variables that are both of maximal variance, and hence, summarize the information included in the raw matrix, and orthogonal. A leave-one-out cross-validation was finally applied to test the predicting power of the analysis. Analyses were done including and excluding species with less than five specimens. No significant differences were revealed between both analyses (results not shown), so analyses including all species were retained.

To test for differences among morphospecies for each quantitative variable, univariate analyses of variance (ANOVA) were first performed to check the global significance for each of the 21 quantitative variables, followed by a post-hoc Tukey test of multiple comparisons of group means. Finally, descriptive statistics were computed for all quantitative variables for each of the resulting species and then summarized in the form of beanplot graphs (Kampstra, 2008), representing the empirical density shape, mean, and all individual observations for each of the recovered morphotypes. The results of post-hoc test were illustrated by different letters per group in the beanplot graphs. These analyses as well as DFA were implemented using R v.3.3.1 (R Core Team, 2016).

Results

Phylogenetic reconstruction and molecular species hypotheses

The amplification of both nuclear loci was not always successful and only 83 sequences were obtained for the *EST*-115 and 71 for *EST*-317, from the total 115 sequences. For both chloroplast loci, *rps*4 and *rpl*32-*trn*L$^{(UAG)}$, 115 and 101 sequences were obtained respectively. The nuclear and chloroplast regions investigated exhibited contrasting levels of variation, including 38 for EST-115 (526 bp), 23 for EST-317 (425 bp), 12 for *rps*4 (670 bp), and 18 *rpl*32-*trn*L$^{(UAG)}$ (617 bp) variable positions among the ingroup species. The resulted final alignment for the four loci combined contained 64 sequences, and 2225 positions, 433 of which are variable and 204 are parsimony informative.

Both ML and BI analyses resolve *Lewinskya affinis* as polyphyletic within a monophyletic clade including also *L. tortidontia* and *L. praemorsa* (Venturi) F.Lara, Garilleti & Goffinet (hereafter *L. affinis* complex) sister to *L. speciosa* (Fig. 3.4.2). The *L. affinis* complex is divided in three well supported clades: one with specimens from Europe, Macaronesia, Africa, and Asia ("Old World", OW clade, BS = 89, PP = 1.0); and two clades with specimens from western North America ("New World", NW clade A, BS=96, PP=1.0; NW clade B, BS=63, PP=0.86). The OW clade includes the lineage of *L. tortidontia* (OW1 BS=78, PP=1.0), a lineage with samples from Europe, North Africa and southwest Asia (OW2, BS=64, PP=0.97), and another highly supported lineage (BS=100, PP=1.0) including a Canarian lineage (OW4) and other one with samples from Europe and East Africa (OW3, PP=1, BS=0.95). The NW clade A is composed by two lineages, the one of *L. praemorsa* (NW2), and other that includes samples from Nevada, Montana, and inner regions of California (NW1, BS=98, PP = 1.0). The NW clade B is also divided in two well-supported lineages, both including specimens from oceanic areas of Oregon, Washington, and British Columbia (NW3 and NW4, both BS=100, PP = 1.0).

Only the multiple threshold GMYC analyses showed significant results for the likelihood ratio test (sGYMC, P = 0.066: mGMYC, P = 0.041) and we refer to it hereafter. The mGMYC analyses delimited eleven putative genetic entities within the *Lewinskya affinis* complex: 1) four lineages from western North America, one corresponding to *L. praemorsa*; 2) an entity restricted to Macaronesia; 3) *L. tortidontia*, which is divided in two lineages; and 4) the OW clade, which is split in four lineages, two corresponding to the OW2 lineage and other two to the OW3 (Fig. 3.4.S1). The bGMYC suggested the same four lineages for

western North America and Macaronesia, maintained the same split for the OW2 lineage, but identified *L. tortidontia* as only one lineage, and unified the OW3 lineage (Fig. 3.4.S1).

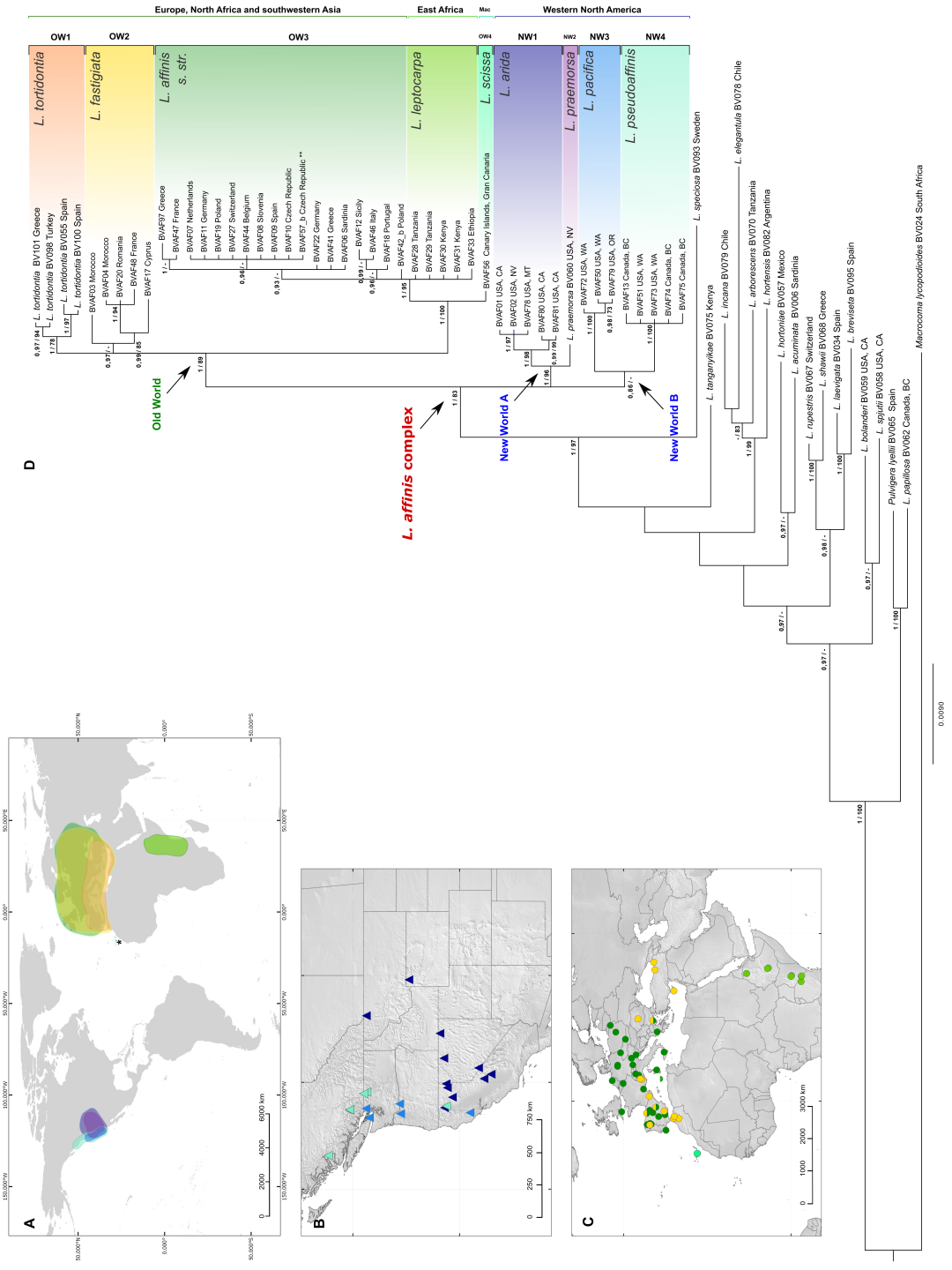

Figure 3.4.2. A: Maps with the general distribution of the nine species of the *Lewinskya affinis* complex; B: distribution of the samples used in the study of *L. affinis s.l.* for the New World and C: the Old World. D: Majority-rule consensus tree obtained in the Bayesian analysis. Bayesian posterior probabilities (PP≥ 0.85) and maximum likelihood bootstrap values (BS≥ 70%) are shown above branches (PP/BS). The different colors represent each of the nine species of the *L. affinis* complex according to the best model resulted from Bayes Factor Delimitation analyses. OW = Old World, NW = New World, Mac = Macaronesia. ** = type material of *Orthotrichum affine* var. *bohemicum*.

Morphological analyses

In the PCA analysis of quantitative characters, the first three principal components (PCs) account for 56.4% of the variance (Figs. 3.4.3A and 3.4.S2). Samples exhibit a continuous range of morphological variation and are intermingled without any clear grouping structure, except for three samples of western North America with outlier values for leaf costa traits, and a group of specimens from Europe, North Africa and Asia that appear almost individualized from the rest of samples with positive values for PC1 and PC2. The most important variables in the first three PCs refer to leaf characters and exothecial band width (Table 3.4.S3). In PC1, the characters with higher correlation values are upper leaf length and perichaetial leaf length and width, thus referring to leaf size. In PC2, the most important variables are acumen length in upper and perichaetial leaves and costa width at central lamina and base, and capsule exothecial band width. In PC3, leaf costa width at base and exothecial band cells length are the most important variables.

Regarding qualitative PCA analysis (Figs. 3.4.3B and 3.4.S3), the first three principal components account for 35.7% of the variance. Here, at least four groups of samples are revealed. The specimens of East Africa occupy a well-defined position with negative coordinates along PC1 and around cero in PC2. This group is opposed to other large group that include specimens from Europe, North Africa, Asia and North America. Additionally, other group of specimens from western North America is clearly individualized with negatives values for PC1 and positive values for PC2, neatly opposed in the second axis to a fourth well-defined group with specimens from Macaronesia. The most important variables for each principal component (Table 3.4.S4) are: in PC1, perichaetial leaf shape ovate-lanceolate (ID=13), exothecial bands clearly not reaching the mouth (ID=39), and exothecial bands only differentiated in the first third of the urn (ID=40); in PC2, perichaetial leaf apex acuminate (ID=17), calyptra with few hairs (ID=21), and calyptra with very abundant hairs (ID=23); and in PC3 vegetative leaf margins revolute or recurved (ID=10), and capsules moderately (ID=37) and deeply furrowed (ID=38).

A clearer grouping emerges in both PCA analyses when the samples are represented according to geography. Samples from different areas frequently overlap, whereas samples living in sympatry always form individualized groups. This grouping is better reflected when information from molecular analyses is included, especially in the qualitative PCA. Then, the analyses show a certain degree of morphological differentiation among the six lineages recovered within *Lewinskya affinis s.l.* in the phylogeny and GMYC analyses (Fig. 3.4.3).

This also supports the split of the lineage OW3 between samples from Europe, North Africa and Asia, and those materials from East Africa (Fig. 3.4.3B).

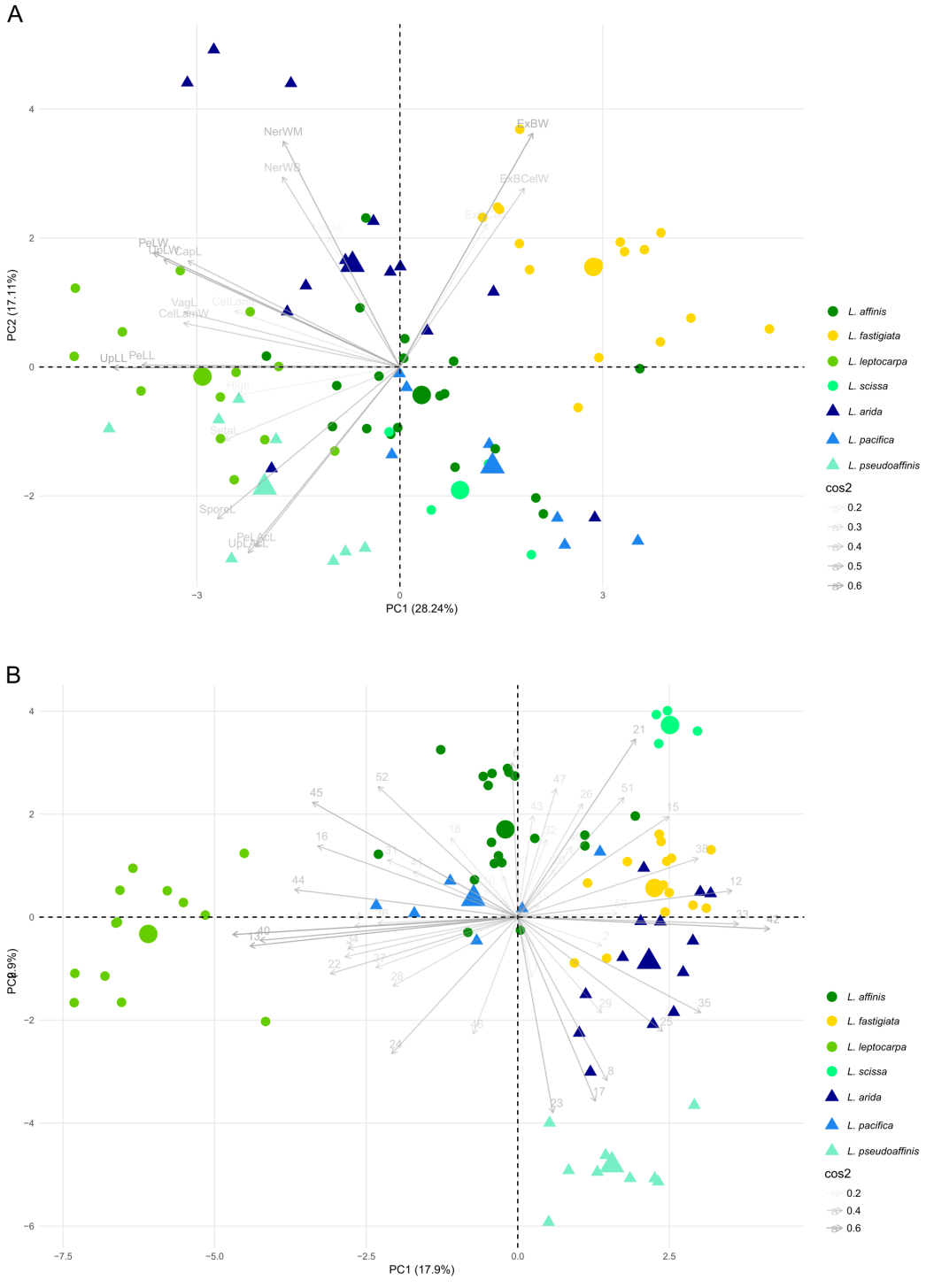

Figure 3.4.3. Principal component analyses (PCA) of *Lewinskya affinis s.l.* representing the first two principal components. A: Quantitative traits. B: Qualitative traits. Variables and their importance for each axis (cos2) are represented as arrows, but see also Table 3.4.S2. Samples are colored following the best hypothesis model obtained in BFD analyses. Circle = Old World, triangle = New World. The larger circles and triangles correspond to the centroid of each group.

The resulting morphological hypothesis to test in BFD analyses would be nine morphospecies (H9, see below), one for each of the six lineages resolved by molecular analyses together with the East African group revealed by morphological PCA analyses for *Lewinskya affinis s.l.*, plus the two already described species *L. tortidontia* and *L. praemorsa* that are included within the clade of the *L. affinis* complex.

Species delimitation and validation

Marginal likelihood values for alternative species delimitation models assessed using BFD favor a nine species model (H9; Table 3.4.2, Fig. 3.4.S4). This model refers to the complete clade of *Lewinskya affinis* complex (Figs. 3.4.2, 3.4.S1), including *L. tortidontia* and *L. praemorsa* together with seven species within *L. affinis s.l.*: *L. affinis s.str.*, *L. fastigiata* (Bruch ex Brid.) Vigalondo, F.Lara & Garilleti, *L. leptocarpa* (Müll.Hal.) Vigalondo, F.Lara & Garilleti, and *L. scissa* sp. nov. for the Old World, together with *L. arida* sp. nov., *L. pacifica* sp. nov., and *L. pseudoaffinis* sp. nov. for the New World. The closest models are the eight (H8) and seven species model (H7), both derived from phylogenetic hypotheses, with a 2lnBF slightly bigger than 10. Both of these models imply the same morphospecies except that model H8 reflects the split of *L. affinis* into two morphospecies, including *L. scissa* for Canary Islands. None of them included *L. leptocarpa*, only tested in model H9.

A DFA was performed to test the validity of the species proposed by the best hypothesis according to BFD analyses (H9), but considering only the seven species within *Lewinskya affinis s.l.*, since the other two of the *L. affinis* complex, *L. tortidontia* and *L. praemorsa*, are already well characterized morphologically and nowadays accepted as different species (Lara *et al.*, 2016). The results of the DFA suggest that the seven species identified by the H9 model can be, to a large extent, distinguished morphologically, being the overall correct classification rate following cross-validation of 87.6%. The probability of correctly assign specimens of *L. leptocarpa*, *L. pseudoaffinis* and *L. scissa* based on morphological traits alone is maximal (100%), being the classification rate of *L. affinis s.str.*, *L. fastigiata*, *L. scissa*, *L. pacifica* and *L. arida* somewhat lower (71.4–78.9%) (Table 3.4.3). The DFA also reveals that, while allopatric species sometimes overlap morphologically (*L. affinis s.str.* with *L. pacifica*, and *L. arida* with *L. fastigiata*), a clearer differentiation among sympatric species emerges (Fig. 3.4.S5).

Table 3.4.3. Classificatory matrix from discriminant function analysis (DFA) for the seven species within *Lewinskya affinis s.l.* based on original and cross-validated grouped cases.

			Predicted Group Membership							
Species			*L. aff*	*L. fas*	*L. lep*	*L. sci*	*L. ari*	*L. pac*	*L. pse*	Total
Original (a)	Count	*L. affinis*	16	1	0	0	0	2	0	19
		L. fastigiata	0	16	0	0	0	0	0	16
		L. leptocarpa	0	0	13	0	0	0	0	13
		L. scissa	0	0	0	4	0	0	0	4
		L. arida	0	2	0	0	11	0	0	13
		L. pacifica	1	0	0	0	0	6	0	7
		L. pseudoaffinis	0	0	0	0	0	0	9	9
	%	*L. affinis*	84,2	6,3	0	0	0	28,6	0	100
		L. fastigiata	0	100	0	0	0	0	0	100
		L. leptocarpa	0	0	100	0	0	0	0	100
		L. scissa	0	0	0	100	0	0	0	100
		L. arida	0	12,5	0	0	84,6	0	0	100
		L. pacifica	5,3	0	0	0	0	85,7	0	100
		L. pseudoaffinis	0	0	0	0	0	0	100	100
Cross-validated (b)	Count	*L. affinis*	15	0	0	1	0	3	0	19
		L. fastigiata	0	15	0	0	1	0	0	16
		L. leptocarpa	0	0	13	0	0	0	0	13
		L. scissa	0	0	0	4	0	0	0	4
		L. arida	0	2	0	0	10	0	1	13
		L. pacifica	1	0	0	1	0	5	0	7
		L. pseudoaffinis	0	0	0	0	0	0	9	9
	%	*L. affinis*	78,9	0	0	25,0	0	42,9	0	100
		L. fastigiata	0	93,8	0	0	7,7	0	0	100
		L. leptocarpa	0	0	100	0	0	0	0	100
		L. scissa	0	0	0	100	0	0	0	100
		L. arida	0	12,5	0	0	76,9	0	11,1	100
		L. pacifica	5,3	0	0	25,0	0	71,4	0	100
		L. pseudoaffinis	0	0	0	0	0	0	100	100

(a) 92.6% of original grouped cases correctly classified. (b) 87.6% of cross-validated grouped cases correctly classified.

Post-hoc test for the quantitative characters reflects differences among the seven species for all variables (Fig. 3.4.S6), and reveals that four of the seven species show unique variables that are significantly different from the other ones. *Lewinskya fastigiata* differs in the exothecial band width and exothecial band cell width, *L. leptocarpa* in perichaetial and upper leaf width and spore diameter, *L. pseudoaffinis* in perichaetial leaf acumen length, and *L. arida* in exothecial band width. Regarding qualitative traits, each of the seven newly delimited species, together with the two already recognized *L. tortidontia* and *L. praemorsa*, can be defined by an independent combination of characters, mainly related with the shape and margin curvature of the leaves, leaf apex shape, prominence of capsule furrows, width and cell rows number of the capsule exothecial bands, peristome structure, calyptra hairiness, spore size and ornamentation (see Key to the species).

Discussion

The integrative approach performed here reveals that the current concept of the widely distributed moss *Lewinskya affinis s.l.* includes seven different species that are well characterized molecularly, morphologically, and geographically. In fact, none of the newly recognized species of the *L. affinis* complex, exhibits a trans-continental range since all of them are restricted to either the Old World or the New World (Fig. 3.4.2). We propose here: 1) the re-circumscription of *L. affinis s.str.* for Europe, North Africa and southwestern Asia; 2) the restoration and combination of two names, actually considered as synonyms of *L. affinis*, namely *L. fastigiata* from Europe the Mediterranean, and *L. leptocarpa* from East Africa; and 3) four new species: *L. scissa*, from Canary Islands, plus *L. arida*, *L. pacifica* and *L. pseudoaffinis* from western North America. Our results have substantial implications for the understanding of bryophyte distribution, diversity, and speciation patterns, and add new data to the growing body of evidence for the need to split broadly defined bryophyte species with large distribution ranges into smaller entities with narrower ranges (Hutsemékers *et al.*, 2012; Medina R. *et al.*, 2012, 2013; Hedenäs *et al.*, 2014; Heinrichs *et al.*, 2015; Patiño & Vanderpoorten, 2015; Köckinger & Kučera, 2016; Patiño *et al.*, 2017).

Integrative taxonomy and species delimitation of the *Lewinskya affinis* complex

The morphological similarity of the species integrating the *Lewinskya affinis* complex have led to the traditional broad taxonomical concept of *L. affinis s.l.* In fact, to this taxon have been ascribed almost all *Orthotrichum* like mosses showing the following combination of traits: superficial stomata, capsule immersed to emergent and furrowed, exostome of 8 teeth pairs, reflexed when dry, endostome of 8 or 8+n linear segments, and leaves acute or acuminate, with recurved margins. Only those similar species having additional noteworthy and particular characters were separated from this jumble (Lewinsky, 1993).

The resemblance of *Lewinskya affinis s.str.* with the other species of the complex is revealed by the morphometric quantitative analyses performed here, since this species has a central placement respect to the rest of taxa for quantitative characters (Fig. 3.4.3), and no quantitative (Fig. 3.4.S6) or qualitative character is significantly different only for *L. affinis s.str*. However, despite the mentioned similarities and even for the more variable characters, we have found species-specific differences at qualitative and quantitative level that allow the morphological differentiation of the different species recovered by the best species

delimitation hypothesis based on molecular and morphological data (Table 3.3.2). As stated previously, every species can be segregated by a different combination of characters, being the most noteworthy the number of cell rows and width of the exothecial band, leaf apex shape, leaf acumen length and leaf width, calyptra hairiness, capsule position respect to perichaetial leaves, exostome structure, and spore size and ornamentation. Different authors already mentioned some of these characters, especially for the differentiation of *L. fastigiata* (Frahm, 2011 for review; Nyholm, 1956; Lara & Garilleti, 2014), but in most cases considering them too variable as to be taxonomically conclusive or even relevant (Vitt, 1973; Lewinsky, 1978, 1998).

Lewinskya affinis s.str. is characterized by: 1) lanceolate leaves with acute or apiculate, usually asymmetric apex; 2) capsules emergent, cylindrical or little contracted below mouth, with thin but marked ribs along its length; 3) exothecial bands narrow of 2-3 cells wide, neatly almost reaching the mouth; 4) exostome teeth not cancellate in the apex; 5) calyptra scarcely hairy; and 6) spores with thin and irregular papillae. This species is mainly restricted to Europe, although present in northernmost Africa and Turkey. Asiatic materials cited by Enroth *et al.* (2016) and revised for this study actually belong to other species. Furthermore, Ignatov *et al.* (2006) indicated that records of *L. affinis* from Kamchatka are dubious and those from southern Russian Far East were actually *L. sordida* (Sull. & Lesq.) F. Lara, Garilleti & Goffinet. In some cases, we have found specimens showing an endostome of 16 segments, or eight plus remains of other intermediary segments. Plášek *et al.* (2011) described *Orthotrichum affine* var. *bohemicum* Plášek & Sawicki from Central Europe, mainly morphologically differentiated from *L. affinis* by the presence of 16 endostome segments. Our molecular results place the type specimen and other samples studied of this variety within the lineage of *L. affinis s.str.* (Fig. 3.4.2). Besides they are neither segregated in the morphological analyses performed, and in consequence we interpret this taxon as a mere form of *L. affinis s.str.* The number of endostomial segments is a variable character in this and other species of the *L. affinis* complex, as intermediate segments can be present and more or less developed, similarly to many other *Orthotrichum* and *Lewinskya* species (Lewinsky-Haapasaari & Hedenäs, 1998; Lara & Garilleti, 2014).

Lewinskya fastigiata is rescued here and combined at species level. The similarity between the basyonyms *Orthotrichum affine* and *O. fastigiatum* was already mentioned in the description of the latter (Bridel, 1827), as well as by other authors that considered both at specific level (e.g. Müller, 1849; Nyholm, 1956). That was also the reason why other

authors treated *L. fastigiata* at different infraspecific levels (e.g. Hübener, 1833; Wijk *et al.*, 1964) or as a mere synonym of *L. affinis* (e.g. Lewinsky, 1998). However, our molecular results clearly separate the lineages of *L. fastigiata* and *L. affinis s.str.*, which are also well supported by morphological differences, and agrees with the recent suggestions of Frahm (2011) and Lara & Garilleti (2014) about the differentiation of both taxa. *Lewinskya fastigiata* differs from *L. affinis s.str.* by having shorter and more broadly lanceolate leaves, capsule deeply furrowed, exothecial bands of four rows of wider cells, exostome teeth clearly cancellate in the apex and ornamented with radial or vermiculate lines, and spores verrucose and usually with scattered coarse lines (type II sensu Medina N.G. *et al.*, 2009). Our results agree with the observations of several authors that noted the broader ribs and exothecial bands of the capsule as one of the most remarkable characters of *L. fastigiata* (Mönkemeyer, 1927; Nyholm, 1956; Frahm, 2011; Lara & Garilleti, 2014). Some of them also considered differences in spore size although, as Frahm (2011) suggested, size is not a distinguishing character according to our results. Nevertheless, as Lara & Garilleti (2014) noticed, spore ornamentation is clearly different between *L. fastigiata* and *L. affinis s.str.*, being each of the two types of spore found for *L. affinis s.l.* (Medina N.G. *et al.*, 2009) characteristic of one of the two species. The distribution range of *L. fastigiata* overlaps to a large extent with that of *L. affinis s.str.*, the first reaching further south in North Africa. Moreover, in many occasions both species can be found growing together, sometimes forming mixed cushions.

We also propose the reinstatement of *Lewinskya leptocarpa* at species level, with a distribution restricted to East Africa. Although specimens of *L. leptocarpa* appear in the phylogeny and according to GMYC analyses within the lineage of *L. affinis s.str.*, morphological analyses sustain a clear differentiation as to recognize it as a different species (Figs. 3.4.3, 3.4.S4, 3.4.S6, Table 3.4.4). Furthermore, BFD analyses support the split of *L. leptocarpa* from *L. affinis s.str.* within the best model hypothesis (Table 3.4.2), and the geographical isolation of these individuals respect to *L. affinis s.str.* can also justified its circumscription. *Lewinskya leptocarpa* differs from the rest of species within the *L. affinis* complex by the presence of very diffuse and short exothecial bands, which are usually neatly separated from the mouth of the capsule by a clear and wide ring of short cells, which sometimes can be noticed even in dry conditions. Other differences with *L. affinis s.str.* are the much broader leaves, ovate-lanceolate the perichaetial ones, with broadly revoluted margins, calyptra strongly hairy with long hairs, and larger spore size. This last character

was the only highlighted by Lewinsky (1978) when she considered *L. leptocarpa* as synonym of *L. affinis s.l.*

Regarding the newly discovered species, all of them are clearly identified as independent lineages by molecular analyses (Figs. 3.4.1 and 3.4.S1). Quantitative PCA analyses revealed their similarity with *Lewinskya affinis s.str.*, but the PCA of qualitative traits separate better each of the species from *L. affinis s.str.*, except for *L. pacifica*, and moreover, they are also differentiated among each other (Figs. 3.4.3 and 3.4.S4, Table 3.4.4). Respect to the Canarian *L. scissa*, just found until now on Gran Canaria Island, the main differences with *L. affinis s.str.* are the exostome teeth pairs usually splitting after the detachment of the lid, capsules completely immersed, which are deeply furrowed and urceolate (constricted bellow mouth), and frequently acuminate perichaetial leaves. The other three species, *L. arida*, *L. pacifica* and *L. pseudoaffinis*, have an overlapping distribution along western North America. Grout (1935) and Vitt (1973) in their respective works on North American mosses, considered *L. affinis s.l.* as a variable taxon regarding leaves traits. Our study reveals that this variation actually hide the diversity of species recovered here, since the three new species differ from *L. affinis* s. s.tr., but also among themselves, by the leaves shape and particularly by leaves apex shape. *Lewinskya pseudoaffinis* is the best differentiated in qualitative PCA and DFA analyses (Figs. 3.4.3 and 3.4.S4), mainly due to its acuminate leaves, together with its long emergent capsules and cancellate exostome apex. *Lewinskya arida* shows very broad leaves, somewhat narrowed in the upper part, with broadly revolute margins, apex frequently acute or acuminated but relatively variable, and also has wide exothecial bands. Finally, *L. pacifica* is the most similar to *L. affinis s.str.* according to morphometric analyses (Figs. 3.4.3 and 3.4.S4), but clearly differs from it and the rest of species by having leaves with blunt and symmetric apex.

Molecular analyses also revealed the already recognized *Lewinskya tortidontia* and *L. praemorsa* as independent lineages within the *L. affinis* complex clade. Furthermore, morphological differences of both taxa are also clear respect to the other species of the *L. affinis* complex, as is reflected in the key for the identification of the species (see below).

In line with the raising idea of cryptic speciation in bryophytes revealed by molecular evidence (Heinrichs *et al.*, 2011; Dong *et al.*, 2012; Yu *et al.*, 2013; Aranda *et al.*, 2014; Draper *et al.*, 2015; Buczkowska *et al.*, 2016), the seven-species newly revealed within *Lewinskya affinis s.l.*, could not have been fully solved based on morphological traits alone, but neither using only molecular methods. Here we have employed three main criteria for

the species delimitation: phylogenetic signal, diagnostic morphology and biogeographic consistency. Molecular analyses alone (ML, BI and GMYC analyses) would have segregated only six of the seven species within *L. affinis s.l.*, since *L. leptocarpa* was resolved within *L. affinis s.str.* clade (Figs. 3.4.1 and 3.4.S1), but morphological analyses, especially the qualitative one, allowed the individualization of both taxa (Fig. 3.4.3). Even if all of the seven species correspond to very alike mosses, discriminant analyses looking for the best combination of morphological traits separating them indicated that each can be identified morphologically with a minimal error rate (Table 3.4.S4). Interestingly, while allopatric species somewhat overlap in the morphological space (*L. affinis s.str.* with *L. pacifica*, and *L. fastigiata* with *L. arida*), sympatric species are more clearly separated morphologically (Fig. 3.4.S4), allowing to better recognize each of them.

Our study thus supports that for a more accurate and robust species delimitation, several methods should be integrated, not only different genetic methods (Carstens *et al.,* 2013), but also different sources of data, with particular importance for morphological information (Edwards & Knowles, 2014), and test their congruence by validation analyses. Furthermore, statistical morphometric analyses have resulted a very informative tool as a complement of traditional qualitative taxonomic approaches, supporting or highlighting the significance of some characters for the discrimination of the species. Integrative taxonomy also revealed the existence of several species within *Orthotrichum consimile* Mitt. (Medina R. *et al.,* 2012) and *O. tenellum* Bruch ex Brid. (Medina R. *et al.,* 2013). In both cases, the overall morphological similarity shared by the discovered species agreed with a process of convergent evolution, since the species belonged to different lineages. On the contrary, the resemblance of the species of the *L. affinis* complex is congruent with the fact that they are sibling species as members of a single monophyletic group (Bickford *et al.*, 2007).

The finding of seven species within *Lewinskya affinis s.l.* presented here, together with similar evidence for the presence of six species within *Orthotrichum tenellum* (Medina R. *et al.,* 2013), four species within *O. consimile* (Medina R. *et al.,* 2012), 10 species in *Frullania tamarisci* (Heinrichs *et al.,* 2010), seven species within *Radula buccinifera* (Renner *et al.,* 2013), or three species within *Homalothecium sericeum* (Hedenäs *et al.,* 2014), suggests that the rate of discovery of new bryophyte species combining molecular and morphological evidence, largely exceeds the rate of synonimizations. In line with this, Renner *et al.*, (2017) concluded for the liverwort genus *Plagiochila* that "real diversity is 29% higher than currently recognized", and that "36%, of currently accepted and previously untested

Australasian species have circumscription issues, including polyphyly, paraphyly, internal phylogenetic structure, or combinations of two or more of these issues". Hence, our study provides additional support to the idea that the actual diversity of bryophyte species could be largely underestimated, and underlines the utility of integrative taxonomy to assess biodiversity.

Biogeography and species diversification

The narrow geographic range of the seven species discovered (Fig. 3.4.1), contrasts with the widespread distribution of the up-to-date concept of *Lewinskya affinis s.l.* This fact is reminiscent of the cases of *Orthotrichum tenellum*, wherein the segregate species recently described are endemic to either the Mediterranean-European region or North America (Medina R. *et al.*, 2013), and the liverworts *Metzgeria conjugata* Lindb. and *M. furcata* (L.) Corda, whose phylogeography is sharply divided into North American and European lineages (Fuselier *et al.*, 2009). This situation has been also described for bryophytes displaying other disjunct distributions (e.g. Hedenäs *et al.*, 2014; Heinrichs *et al.*, 2015; Scheben *et al.*, 2016, Patiño *et al.*, 2017), suggesting that these patterns contrast with the traditional perception that bryophyte species exhibit large, transoceanic distribution ranges (for review see Shaw 2001, Vanderpoorten *et al.*, 2010) and reflect that the dispersal capacities of these species might be much lower than a broad concept of these species would suggest. In fact, examination of recent bryophyte phylogenies indicates that they are highly structured geographically (Dong *et al.*, 2012, Norhazrina *et al.*, 2016, Scheben *et al.*, 2016, Bechteler *et al.*, 2017). This geographic structure does not contradict the idea that many disjunctions observed within or among sister bryophyte species are due to long-distance dispersal (for review see Carter *et al.*, 2017), but indicate, in line with evidence for dispersal limitations inferred from the spatial structure of genetic variation among transoceanic bryophyte populations (Désamoré *et al.*, 2016, Kyrkjeeide *et al.*, 2016), that dispersal may not routinely occur after the speciation process.

The existence of seven species within *Lewinskya affinis s.l.* suppose the split of a largely distributed bryophyte species into sister species with narrower ranges that do not span several continents. Thus the regional endemicity of these seven species, as well as in other Orthotrichaceae (Medina R. *et al.*, 2012, 2013), and other bryophytes (e.g. Hedenäs *et al.*, 2014; Patiño *et al.*, 2017), suggests that the extremely low rates of endemism documented in bryophytes by comparison with angiosperms (Vanderpoorten *et al.*, 2010) could largely

be due to an artifact. In this regard, the description of the Canarian new endemic species *L. scissa* is significant as only 2% of the moss species were considered Canarian endemics to date (González-Mancebo *et al.*, 2008, Vanderpoorten *et al.*, 2011).

The presence within *Lewinskya affinis s.l.* of three monophyletic western North American species on the one hand, and four monophyletic Old World species on the other was masked for the previously considered morphological uniformity of *L. affinis* (i.e. Lewinsky 1998), and involves that there is a tendency for within-continent diversification rather than recurrent anagenesis. Based on evidence for cryptic diversification within continental areas in *Ceratolejeunea* (Spruce) J.B.Jack & Steph., Scheben *et al.*, (2016) suggested that morphological uniformity may mask actual radiations in bryophytes. According to that, the high rates of anagenesis reported for island bryophytes based on the number of endemic species (Patiño *et al.*, 2014) may be, therefore, a gap on taxonomical knowledge. However, recent phylogenetic evidence in *Rhynchostegiella* (Schimp.) Limpr. (Patiño and Vanderpoorten, 2015), points to the polyphyletic origin of the Macaronesian endemic species in the genus and to recurrent patterns of anagenetic speciation. One hypothesis for the contrasted patterns of in-situ diversification in island and mainland bryophyte species is that, as Kisel and Barraclough (2010) proposed, speciation has a spatial scale that depends on levels of gene flow, and hence, that many oceanic islands may be too small to allow efficient dispersers like bryophytes to diversify.

Taxonomic treatment

All the species of the *Lewinskya affinis* complex are phaneroporous Orthotricheae that share the following relevant characteristics: small to medium size mosses, forming cushions on bark or exceptionally on rock surfaces; leaves variably lanceolate with recurved to revolute margins in most of their length; capsule immersed to emergent, furrowed when mature; peristome double; exostome of 8 pairs of teeth, recurved after detachment of the operculum; endostome of 8 or 8+n linear segments, smooth in the outer side (PPL) and ornamented in the inner part (IPL) by a reticule variably prominent; operculum rostrate with reddish basal rim differentiate; vaginula naked; calyptra conic-oblong with multiseriate, moderately papillose hairs; spore papillose.

Key to species

1. Exothecial bands broad, 4 rows of cells near capsule mouth (often 6–8 below) 2

1'. Exothecial bands narrow, 2–3 rows of cells near capsule mouth (sometimes 4-6 below).....5

2. Exostome teeth pairs splitting after the detachment of the lid, recurved and irregularly twisted and sometimes overlapping when dry (fragile and most frequently broken in old capsules); capsule mouth star-shaped when dry (zenithal view); exothecial bands frequently differentiated near capsule mouth only (Mediterranean) .....***L. tortidontia***

2'. Exostome teeth pairs persistent in capsules of the current year; capsule mouth ring-shaped when dry; exothecial bands usually differentiated along whole urn.....3

3. Leaves apex long acuminate and frequently aristate; calyptra strongly hairy, with long hairs evenly distributed (W N Amer.).....***L. praemorsa***

3'. Leaves acute to short acuminate or apiculate, never aristate; calyptra scarcely to moderately hairy with short or not evenly distributed hairs.....4

4. Leaf margins narrowly revolute (less broad than nerve); capsule short urceolate when dry and empty; calyptra with short or long hairs mostly in its upper third (Europe & Med.).... ***L. fastigiata***

4'. Leaf margins broadly revolute (usually as broad as nerve); capsule cylindrical to long urceolate when dry and empty; calyptra with scattered short hairs (W N Amer.)..... ***L. arida***

5 - Exothecial bands slightly differentiate, short, usually restricted to urn upper third, separated from capsule mouth by a continuous suboral ring of differentiate, short cells; leaves broadly ovate-lanceolate with broadly revolute margins (E Africa).....***L. leptocarpa***

5' - Exothecial bands moderately to strongly differentiate in more than half of the urn, almost reaching the mouth and interrupting the suboral ring of short cells when differentiated; leaves narrowly lanceolate to ovate-lanceolate, with recurved to narrowly revolute margins 6

6. Leaf apex blunt, obtuse to rounded; leaves above base frequently lingulate (W N Amer.)…….. .....***L. pacifica***

6'. Leaf apex acute, apiculate or acuminate; leaves above base gradually narrowed to the apex 7

7. Capsule always immersed, usually well exceeded by perichaetial leaves; exostome teeth usually splitting in capsules of the current year (Canary Is.) ..... ***L. scissa***

7’ - Capsule commonly short to long emergent, sometimes immersed but barely exceeded by perichaetial leaves; exostome teeth not splitting in capsules of the current year 8

8. Leaf acute or apiculate; calyptra scarcely hairy; capsule usually shortly emergent, sometimes immersed or long emergent, never exserted; exostome teeth rarely fenestrate at apex (Europe & Med.) ..... ***L. affinis***

8’. Leaf mostly acuminate; calyptra strongly hairy; capsule hemiemergent to long emergent, occasionally short exserted; exostome teeth clearly cancellate in upper third or upper half (W N Amer.) ..... ***L. pseudoaffinis***

Species descriptions

Lewinskya affinis (Brid.) F.Lara, Garilleti & Goffinet in F.Lara et al. Cryptogamie, Bryol. 37(4): 374. 2016

≡*Orthotrichum affine* Schrad. *ex* Brid., Muscol. Recent. 2: 22–23. 1801

Type: In asseribus et arborum truncis Hassiae et prope Goettingam Gothamque. Types originally at B, not located and probably destroyed in 1943.

Figure: 3.4.4.

Plants 0.4–2.2 mm tall. *Vegetative leaves* 2.0–3.6 x 0.5–0.9 mm, lanceolate, more rarely ovate-lanceolate; leaf apex acute, sometimes apiculate; leaf margins recurved to revolute. *Perichaetial leaves* 3.0–4.6 x 0.6–1.2 mm, ovate-lanceolate; leaf apex usually short apiculate; leaf margins revolute. *Calyptra* scarcely hairy, sometimes moderately hairy, with multiseriate, moderately papillose and mostly short hairs, frequently accumulated in the upper third. *Seta* 0.5–1.3 mm. *Capsule* 1.8–2.8 mm long, immersed or more or less emergent, usually shortly emergent; when dry and empty cylindrical, less frequently oval-cylindrical or urceolate (constricted below mouth); moderately furrowed. *Exothecial bands* of 2–3(4) rows of cells in distal part, 4–6 bellow, moderately to well differentiate in the upper half or in the whole urn; almost reaching the mouth, but separated by 2–3(4) short oblate cells. *Exostome* of 8 pairs of teeth, usually not tending to split after recurving; commonly yellowish-whitish; sometimes weakly fenestrate at apex; OPL ornamented by short vermicular lines of variable orientation and density, sometimes with mixed papillae; PPL open to densely papillose, especially on distal half. *Endostome* of 8 segments, rarely up to 16, with intermediate segments variably developed; IPL ornamented by a line reticule, usually asymmetrically biseriate, sometimes uniseriate, with moderately or slightly thickened transversal walls in the lower half. *Spores* 15–21 μm, papillose, with thin papillae densely disposed, sometimes whit thicker papillae.

Distribution: widespread throughout Europe, reaching Nordic countries, common in Mediterranean mountainous areas. Scarce in North Africa, only found in northernmost areas. Also in south western Asia.

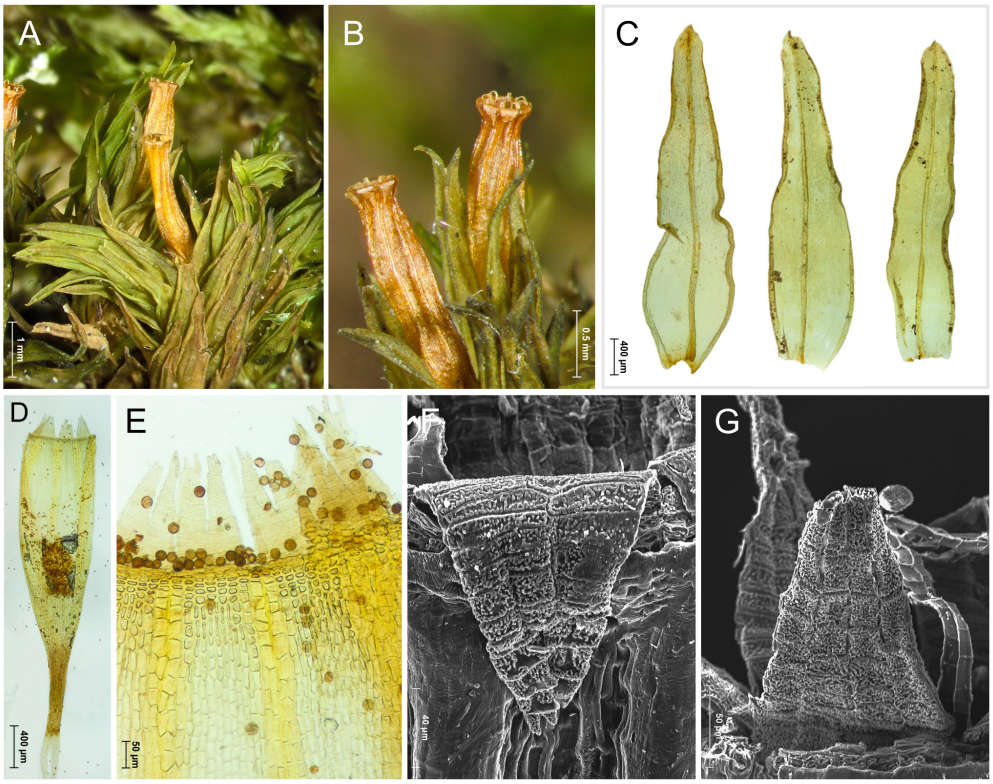

Figure. 3.4.4. *Lewinskya affinis.* A: habit and mature capsules; B: close view of mature capsules; C: perichaetial leaf shape and apex variability; D: dissected sporophyte; E: upper part of the capsule, exotechial bands and peristome; F: exostome PPL, G: exostome OPL, A-B: *Vigalondo & Calleja,* MAUAM 3353; C (left): *Lara, MAUAM 3351;* C (middle and right): *Garilleti 2013-07a & Albertos,* VAL s.n.; D: *Melo,* MAUAM 5047; E: *Lara,* MAUAM 4448; F: *Lara,* MAUAM 1958; G: *Vigalondo & Calleja,* MAUAM 3349.

Lewinskya fastigiata (Bruch *ex* Brid.) Vigalondo, F.Lara & Garilleti, ***comb. nov.***

≡*Orthotrichum fastigiatum* Bruch *ex* Brid. Bryol. Univ. 1: 785. 1827.

Type: In cortice Populi circa Bipontium caespitibus parvulis habitat. Clar. Bruchius detexit et communicavit. Holotype: B 31 0221 01-1! (Zipple 2006).

Figure: 3.4.5.

Plants 0.6–1.8 cm tall. *Vegetative leaves* 2.1–3.1 x 0.5-0.9 mm, lanceolate to ovate-lanceolate; leaf apex acute to short acuminate, frequently asymmetric; leaf margins mostly revolute. *Perichaetial leaves* 2.5–4.1 x 0.6–1.2 mm, usually ovate-lanceolate sometimes lanceolate; leaf apex acute to apiculate, sometimes acuminate, frequently asymmetric; leaf margins revolute. *Calyptra* scarcely hairy, with short hairs frequently accumulated in the upper third. *Seta* 0.3–0.8 mm. *Capsule* 1.7–2.5 mm long, immersed or more or less emergent, usually hemi-emergent; when dry and empty urceolate and constricted below mouth, deeply furrowed. *Exothecial bands* of 4 rows of cells in distal part, 6–8 below; strongly differentiate

in the upper half, less so below, reaching the base of the urn; almost reaching the mouth, but separated by 1–2(4) short oblate cells. *Exostome* of 8 pairs of teeth, usually not tending to split after recurving; yellowish, rarely orange; cancellate and frequently fenestrate at apex; OPL variably ornamented, frequently with a pattern of long lines radially dispose in each cell area, sometimes ornamented with vermicular lines or with small papillae dispersed on a basal reticulum; PPL smooth or faintly ornamented. *Endostome* of 8 segments, rather robust; IPL ornamented by a line reticule, partially or completely biseriate, with moderately thickened transversal or zig-zag medial line walls in the lower half. *Spores* 14-21 μm, ornamented with verrucous and irregular papillae, usually forming irregular coarse bands, sometimes whit thinner papillae.

Distribution: widespread throughout Europe and the Mediterranean Basin, reaching Nordic countries. Common in western North Africa and south western Asia.

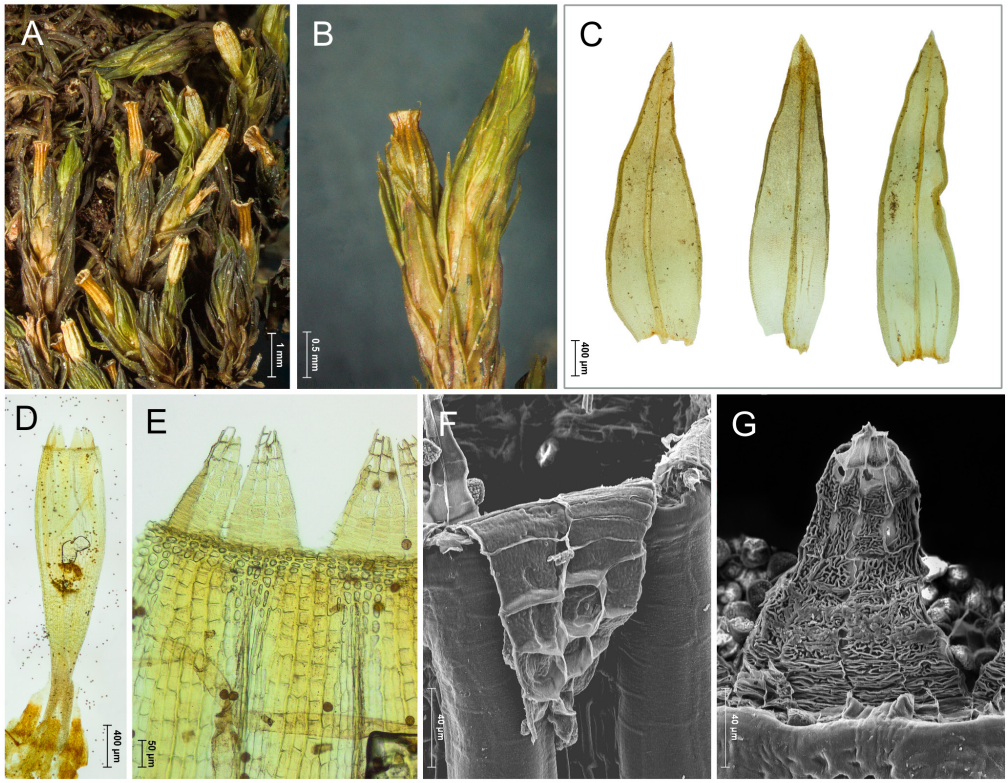

Figure. 3.4.5. *Lewinskya fastigiata*. A: habit with capsules in different stages of development; B: close view of mature capsule; C: perichaetial leaf shape and apex variability; D: dissected sporophyte; E: upper part of the capsule, exotechial bands and peristome, exostome clearly cancellate and fenestrate; F: exostome PPL, G: exostome OPL. A: *Albertos et al.*, MAUAM 2704; B, C (right), E: *Garilleti et al.*, MAUAM 1664; C (left), D: *Lara et al.*, MAUAM 5058; C (middle): *Lara*, MAUAM 5059; F: *Albertos et al.*, MAUAM 2705; G: *Garilleti & Albertos*, VALf 9325.

Lewinskya leptocarpa (Müll.Hal.) Vigalondo, F.Lara & Garilleti, ***comb. nov.***

≡*Orthotrichum leptocarpum* Müll.Hal. Syn. Musc. Frond. 1: 706. 1849.

Type: Abyssinia: W. Schimper legit in monte Silke, ad truncos *Ericae acrophyae*, in reg. sup. ericarum, 16 Febr. 1840. Lectotype: BM 001244102! Here selected. Dixon (1922) specified that the original material of the species was the specimens in half-sheet at Schimper's herbarium (now at BM). A sheet matching this description and annotated by Dixon himself was located in this herbarium. It contains four specimens, only one of these being a pure cushion of *L. leptocarpa*. Lewinsky (1978) followed Dixon's criterion to indicated the lectotype, but she just referred to an imprecise "BM", which makes impossible to know what specimens should be considered as the chosen lectotype. In order to avoid future confusion, we here amend Lewinsky's choice and select as the lectotype of the species the pure specimen of *L. leptocarpa* in the half-sheet indicated by Dixon.

Figure: 3.4.6.

Plants 0.7–2.5 cm tall. *Vegetative leaves* 2.8–3.8 x 0.6–1.2 mm, lanceolate to ovate-lanceolate; leaf apex usually acute; leaf margins recurved to revolute. *Perichaetial leaves* 3.3–5.0 x 0.9-1.5 mm, broadly ovate-lanceolate; leaf apex usually apiculate, frequently with apicule relatively long and asymmetric; leaf margins broadly revolute. *Calyptra* often moderately to strongly hairy, hairs mostly long and clearly accumulated in the upper third. *Seta* 0.4–1.0 mm. *Capsule* 2.0–2.7 mm, immersed or emergent, usually almost hemi-emergent; when dry and empty oval-cylindrical, less frequently cylindrical; moderately furrowed. *Exothecial bands* of 2(3) rows of cells in distal part, 4(6) below, slightly to moderately differentiated only in the upper 1/3(1/2) of the urn; clearly not reaching mouth, frequently separated by a wide suboral ring of short cells. *Exostome* of 8 pairs of teeth, not tending to split after recurving; usually orange or slightly orange; not cancellate or fenestrate; OPL ornamented by vermicular lines of variable length, orientation and density, rarely with mixed papillae; PPL faintly ornamented with short lines and papillae, almost smooth in basal part. *Endostome* of 8 segments, rarely with remains of intermediate ones; IPL ornamented by a line reticule, uniseriate or asymmetrically biseriate, with thickened transversal walls in the lower half. *Spores* 19-28 μm, with thin to thick papillae densely disposed.

Distribution: East Africa. Confirmed for Ethiopia, Kenya and Tanzania.

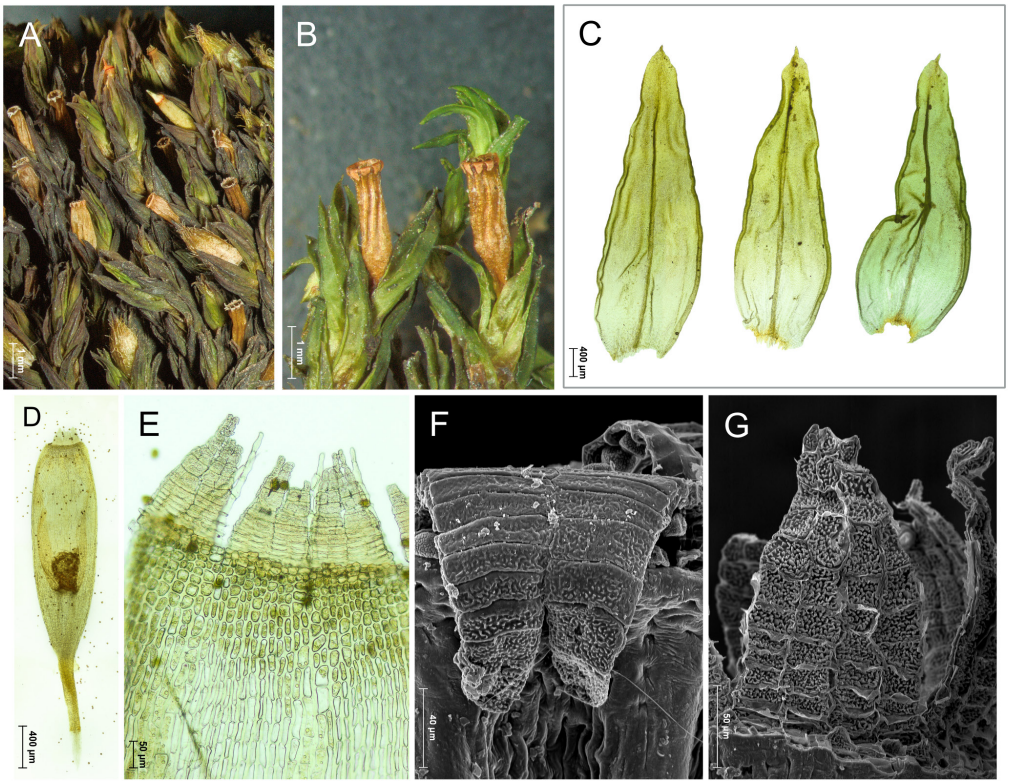

Figure. 3.4.6. *Lewinskya leptocarpa*. A: habit with capsules in different stages of development and calyptrae; B: close view of mature capsules; C: perichaetial leaf shape and apex variability; D: dissected sporophyte; E: upper part of capsule, peristome and notice the diffuse differentiation of the exothecial bands and that they do not reach the mouth; F: exostome PPL, G: exostome OPL. A: *Garilleti et al.*, MAUAM 5068; B, F-G: *Garilleti et al.*, MAUAM 5063; C, E: *Lara et al.*, MAUAM 5064; D: *Vigalondo, Lara & Mazimpaka*, MAUAM 5072.

Lewinskya scissa Vigalondo, F.Lara & Garilleti ***sp. nov.***

Figure: 3.4.7.

Plants 1.0–2.1 cm tall. *Vegetative leaves* 2.5–3.5 x 0.5–0.9 mm, lanceolate to ovate-lanceolate; leaf apex acute to acuminate, rarely asymmetric; leaf margins recurved to revolute. *Perichaetial leaves* 3.7–4.7 x 0.7–1.0 mm, usually ovate-lanceolate, sometimes lanceolate; leaf apex acute to acuminate, rarely asymmetric; leaf margins recurved to revolute. *Calyptra* naked or scarcely hairy, rarely moderately hairy, hairs short when present, frequently accumulated in the upper third. *Seta* 0.3–0.7 mm. *Capsule* 1.6–2.1 mm long, immersed, usually well exceeded by perichaetial leaves; when dry and empty urceolate or cylindrical and somewhat contracted below mouth; deeply furrowed. *Exothecial bands* of 2–3(4) rows of cells in distal part, 4–6 below; well differentiate in the upper 2/3 of the urn; almost reaching the mouth, but separated by 1–3(4) short oblate cells. *Exostome* of 8 pairs of teeth, easily splitting after recurving; yellowish, rarely light orange; not cancellate or

fenestrate; OPL with a contrasting ornamentation, at base with long horizontal lines, above with vermicular short lines and papillae; PPL almost smooth to papillose, less ornamented in basal half. *Endostome* of 8 segments, persistent in old capsules, long, usually thin. IPL strongly ornamented by a line reticule, often partially biseriate, sometimes with moderately thickened transversal walls. *Spores* 16-25 µm, usually ornamented with thin, slightly prominent papillae.

Distribution: Canary Islands. Confirmed for Gran Canaria.

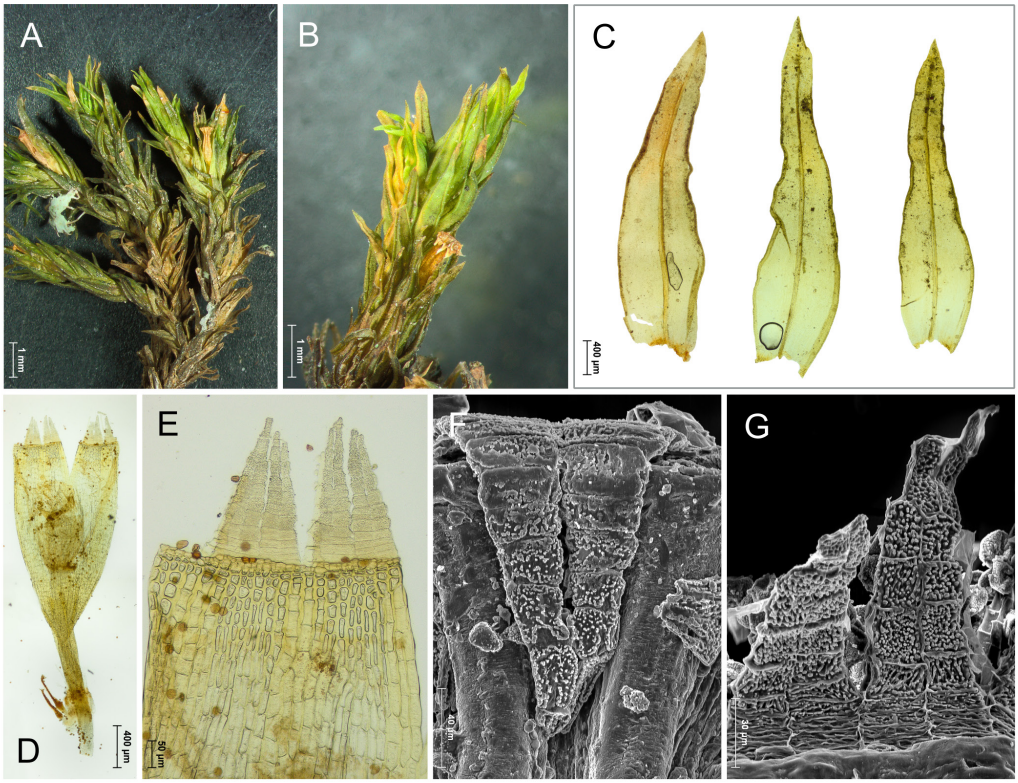

Figure. 3.4.7. *Lewinskya scissa*. A: habit with capsules in different stages of developement; B: close view of immersed capsule; C: perichaetial leaf shape and apex variability; D: dissected sporophyte; E: upper part of the capsule, exotechial bands and peristome, exostome teeth partially split; F: exostome PPL, G: exostome OPL, teeth pair split. A, C (middle and right): *Calleja*, MAUAM 5090; B: *Calleja*, MAUAM 5091; C (left), D-E: *Medina*, MAUAM 5089; F-G: *Vigalondo & Calleja*, MAUAM 5088.

Lewinskya arida Vigalondo, F.Lara & Garilleti ***sp. nov.***

Figure: 3.7.8.

Plants 0.5-1.5 cm tall. *Vegetative leaves* 2.4-3.9(-4.2) x 0.5-1.1 mm, lanceolate to ovate-lanceolate; leaf apex acuminate, acute, apiculate, sometimes blunt, symmetric or asymmetric; leaf margins broadly revolute. *Perichaetial leaves* 3.0-5.0 x 0.7-1.5 mm, mostly ovate-lanceolate, frequently strongly narrowed above base; leaf apex variable as in

vegetative leaves; leaf margins broadly revolute. *Calyptra* scarcely hairy, sometimes naked or moderately hairy, usually with scattered short hairs. *Seta* 0.5-1.2 mm. *Capsule* 1.7-2.6(-3.1) mm long, immersed to emergent, usually hemi-emergent; when dry and empty oval-cylindrical to urceolate, with age becoming long urceolate, deeply furrowed. *Exothecial bands* of 4 rows of cells in distal part, 4-6 (8) below; moderately differentiate in the upper ½ of the urn, slightly differentiate below; almost reaching the mouth, but separated by 1-2 (3) short oblate cells. *Exostome* of 8 pairs of teeth, usually not split after recurving; yellowish; variably fenestrate or cancellate at apex; OPL variably ornamented, frequently with a pattern of lines radially disposed, sometime with short vermicular lines, rarely predominantly papillose; PPL smooth to faintly ornamented in the upper half. *Endostome* of 8 segments; IPL variably ornamented by a line reticule, frequently weakly so, the basal cell always smooth, usually asymmetrically biseriate. *Spores* 13-21 mm, finely papillose, sometimes with somewhat thicker papillae, frequently with papillae fused into irregular bands.

Distribution: western North America. Confirmed for California, Nevada, Montana, Wyoming (USA).

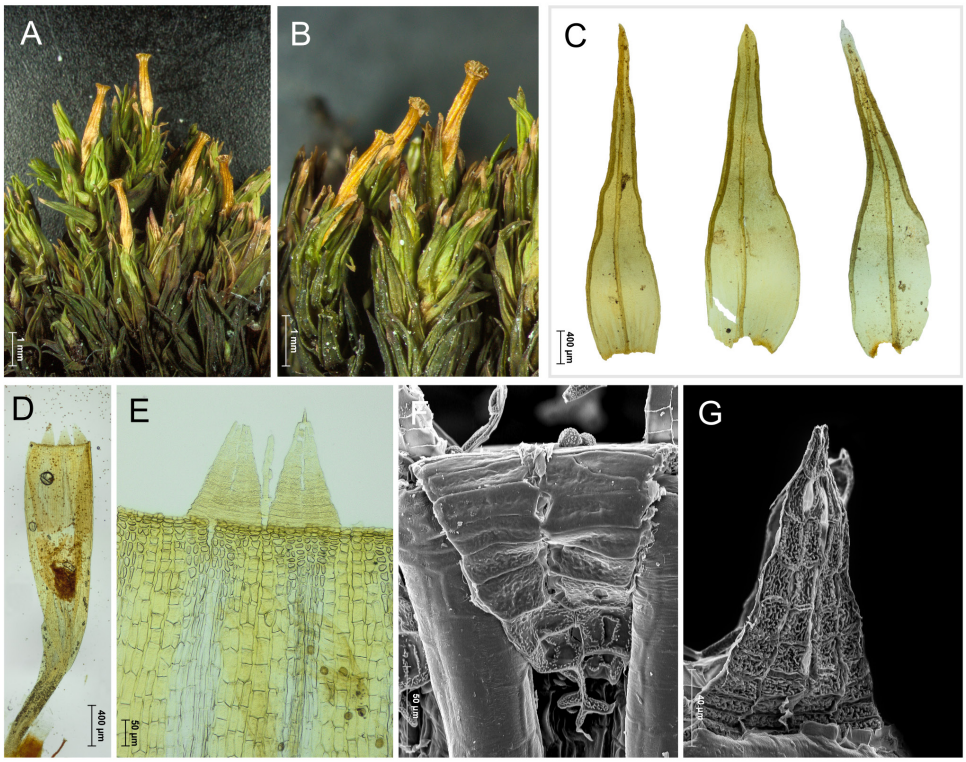

Figure. 3.4.8. *Lewinskya arida*. A: habit and mature capsules; B: close view of mature capsules; C: perichaetial leaf shape and apex variability; D: dissected sporophyte; E: upper part of the capsule, exotechial bands and peristome; F: exostome PPL cancellate, G: exostome OPL. A-B: *Calleja & Vigalondo*, MAUAM 5051; C: *Lara, Garilleti & Albertos*, MAUAM 5053, D: *Lara et al.*, MAUAM 5055; E: *Calleja & Vigalondo*, MAUAM 5052, F-G: *Laeger*, MAUAM 5049

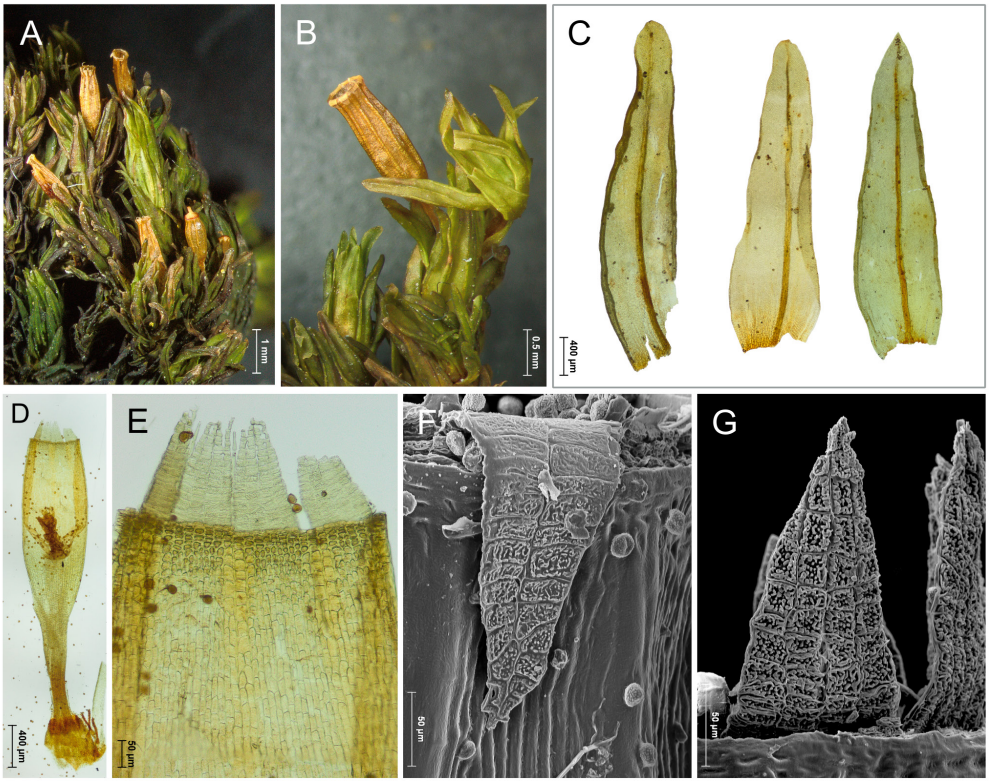

Figure. 3.4.9. *Lewinskya pacifica*. A: habit with capsules in different stages of development; B: capsule, notice the presence of perichaetial leaves with blunt apex; C: perichaetial leaf shape and apex variability; D: dissected sporophyte; E: upper part of the capsule, exotechial bands and peristome; F: exostome PPL, G: exostome OPL. A, D-E: *Lara & Garilleti*, MAUAM 5074; B-C, F-G: *Lara, Garilleti & Albertos*, MAUAM 5075.

Lewinskya pacifica Vigalondo, F.Lara & Garilleti ***sp. nov.***

Figure: 3.4.9.

Plants 0.7-2.2 cm tall. *Vegetative leaves* 2.1-3.3 x 0.5-0.9 mm, narrowly lanceolate to lanceolate, rarely ovate-lanceolate; leaf apex blunt, obtuse to rounded, or acute, usually symmetric; leaf margins recurved to revolute. *Perichaetial leaves* 2.5-4.5 x 0.7-1.1 mm, lanceolate to ovate-lanceolate, with long ovate base, lamina almost ligulate; leaf apex blunt, frequently narrowly rounded, rarely acute or short apiculate, usually symmetric; leaf margins recurved to revolute. *Calyptra* scarcely hairy, rarely moderately hairy, usually with scattered short hairs, rarely accumulated in the upper third. *Seta* 0.5-1.1 mm. *Capsule* 1.5-2.6 mm long, immersed to long emergent, more frequently hemi-emergent; when dry and empty cylindrical, oval-cylindrical or moderately urceolate; moderately to deeply furrowed. *Exothecial bands* of 2-3 (4) rows of cells in distal part, 4-6 below; moderately differentiate in the upper 1/2 of the urn, sometimes slightly differentiate below; almost reaching the

mouth, but separated by 1-4 short oblate cells. *Exostome* of 8 pairs of teeth, usually not split after recurving; usually yellowish; occasionally weakly fenestrate at apex; OPL ornamented with sinuous lines of variable length, usually with scattered papillae; basal remnants of prostome frequently present; PPL smooth. *Endostome* of 8 segments, rarely with some intermediate ones developed, robust; IPL ornamented by a line reticule, mostly biseriate, with transversal or zig-zag medial line walls moderately thickened. *Spores* 13-21 papillose, papillae usually irregular and thick, sometimes thinner.

Distribution: western North America. Confirmed for California, Oregon, Washington (USA), and British Columbia (Canada).

Lewinskya pseudoaffinis Vigalondo, F.Lara & Garilleti ***sp. nov.***

Figure: 3.4.10.

Plants 0.8–2.5 cm tall. *Vegetative leaves* 3.0–3.8 x 0.6–0.9 mm, long lanceolate; leaf apex mostly acuminate, commonly asymmetric; leaf margins usually recurved. *Perichaetial leaves* 3.3–4.8 x 0.7–1.3 mm, long lanceolate to ovate-lanceolate; leaf apex long acuminated, frequently asymmetric; leaf margins recurved to revolute. *Calyptra* strongly hairy, with multiseriate, moderately papillose and long hairs, scattered along the calyptra, not accumulated in the upper part. *Seta* 0.7–1.3 mm. *Capsule* 2.0–2.8 mm long, usually long emergent, sometimes clearly exerted; when dry and empty oval-cylindrical or cylindrical, moderately furrowed, with age becoming long urceolate and deeply furrowed. *Exothecial bands* of 2–3 rows of cells in distal part, 4–6 below; moderately differentiate in the upper ½ of the urn, slightly differentiate below; almost reaching the mouth, but separated by 1–4 short oblate cells. *Exostome* of 8 pairs of teeth, usually not split after recurving; yellowish; clearly cancellate in upper third, sometimes in the upper half; OPL ornamented with thin, sinuous lines in the basal part, short lines and papillae in the upper part; PPL smooth or faintly ornamented. *Endostome* of 8 segments; IPL variably ornamented by a line reticule, sometimes very weakly, usually biseriate, sometimes very asymmetrically so, occasionally with transversal walls strongly thickened. *Spores* 13–21 mm, ornamented with verrucous and irregular papillae, usually forming irregular coarse bands.

Distribution: western North America. Confirmed for California, Oregon, Washington (USA), and British Columbia (Canada).

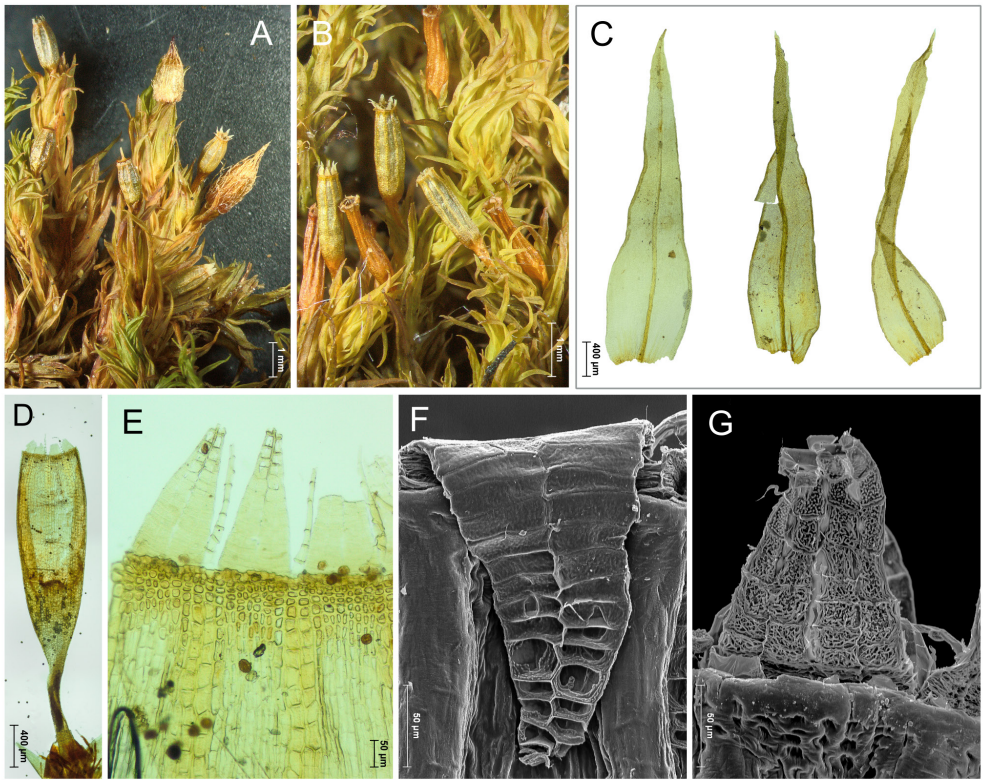

Figure. 3.4.10. *Lewinskya pseudoaffinis*. A: habit with capsules in different stages of development and calyptrae; B: close view of capsules in different stages of development; C: perichaetial leaf shape and apex variability; D: dissected sporophyte; E: upper part of the capsule, exotechial bands and peristome, exostome clearly cancellate at apex and endostome with marked transversal walls; F: exostome PPL, clearly cancellate at apex, G: exostome OPL. A, D: *Lara et al.* MAUAM 5084; B, F-G: *Lara et al.* MAUAM 5080; C: *Lara & Garilleti*, MAUAM 5081; E: *Lara & Garilleti*, MAUAM 5082.

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Appendix

Selection of specimens of the *Lewsinkya affinis* complex used for morphological and molecular analyses, including voucher information. Those included in molecular analyses are followed by the DNA identification code between braquets as it appears in Table 3.4.S5. According to the guidelines, GenBank accession numbers will be provided after the manuscript is accepted.

***Lewinskya affinis* (Brid.) F.Lara, Garilleti & Goffinet.**

Austria: Tirol, End of Ötztal Valley, NW of Ötz, 22.07.2013, *F. Lara 1307/55, MAUAM 3352, [-];* **Belgium:** Wallonia, Between Masbourg and Lesterny, strem close to the railway, 27.08.2013, *Vigalondo & Calleja, MAUAM 3349, [BVAF44];* **Czech Republic:** Bohemia, PLA, Lucické hory Mts., 0,9 km NNW centre of village Doubice, 18.10.2006, *Markóva, OSTR s.n., [BVAF57];* Moravia, Bilà, road 484 to Slovaquia border, 11.08.2013, *Vigalondo et al., MAUAM 3355, [BVAF10];* **France:** Languedoc-Roussillon, Lozère, Les Cévennes, between Puech Arnal and Mandagout, stream of Navés, 21.07.2013, *Garilleti 2013-07a & Albertos, VAL s.n., [BVAF47];* **Germany:** Baviera, Berchtesgaden Land, bridge of Alpenstrasse over the river Schwarzback, 26.07.2013, *Lara, MAUAM 3351, [BVAF22];* Harz Mts., border of the National Park, 19.08.2013, *Vigalondo & Calleja, MAUAM 3347, [BVAF43];* Harz Mts., Königshütte, 19.08.2013, *Vigalondo et al., MAUAM 3356, [BVAF11];* **Greece:** Eastern Makedonia, Mt Falakro, Gorge of Pirgi, NE from Pirgi, 21.06.2007, *Blockeel 34/416, [BVAF97];* Ípiros, Ioánina, Vikos Gorge, between Aristi and Papingo, 30.07.1999, *Lara et al., MAUAM 3345, [BVAF41];* **Italy:** Cerdeña, Monte del Gennargentu, Passo de Caravai, 18.03.2008, *Lara, MAUAM 3343, [BVAF06];* Piemonte, Torino, Alice Superiore, close to camping Valchiusella, 28.07.2013, *Garilleti 2013-31a & Albertos, VAL s.n., [BVAF46];* Sicilia, Peloritani-Nebrodi, road from Floresta to Santa Domenica Vittoria, 08.09.2000, *Lara et al., MAUAM 3354, [BVAF12];* **Netherlands:** Groningen, Anjum, 20.08.2013, *Vigalondo & Calleja, MAUAM 3348, [BVAF07];* **Poland:** P.N. Kampinosky, Parking, 15.08.2013, *Vigalondo & Calleja, MAUAM 3353, [BVAF19];* Ryn, outside the village, 17.08.2013, *Vigalondo & Calleja, MAUAM 3346, [BVAF42];* **Portugal:** Algarve, 13.07.2000, *Melo, MAUAM 5047, [BVAF18];* Tras-os-Montes e Alto Douro, Sierra de Nogueira, pass Lançao, dirty road, 23.11.2000, *García, et al.,, MAUAM 2894, [BVAF37];* **Slovenia:** Jesenice, Triflavski N.P., Ukana, Bohinjsko Lake, 29.07.2013, *Lara, MAUAM 3350, [BVAF08];* **Spain:** Ciudad Real, Cabañeros, 10.10.1993, *Vergara & Lara, MAUAM 1675, [BVAF35];* Jaén, Martos, Sierra de Víboras, 06.04.2012, *Lara, MAUAM 4448, [BV015];* Lugo, Navía de Suarna, Muñís, Casa de Fontela, 02.01.1994, *Albertos et al., MAUAM 1275, [BVAF58];* Lugo, Cervantes, Castelo (San Pedro), 01.01.1994, *Albertos et al., MAUAM 1227, [BVAF62];* Segovia, Riaza, close to Puerto de la Quesera, 02.09.1990, *Lara, MAUAM 1958, [BVAF09];* Zamora,

Puente de Sanabria, *MAUAM 1245, [BVAF61];* **Switzerland:** Valais, Gom, Fiestchertal, 10.08.2013, *Garilleti 2013-101c & Albertos, VAL s.n., [BVAF27];* **United Kingdom:** Lake District, Lake District, Borrowdale valley, 23.07.2003, *Lara, MAUAM 2912, [BVAF36]*.

Lewinskya arida Vigalondo, F.Lara & Garilleti.

USA: California. Lasser Co., 1.5 miles south of the border with Modoc Co., 30.10.2008, *Lara et al., MAUAM 5048, [BVAF52];* California, Fresno Co., Sierra Nat. Forest, along Big Creek at Blue Canyon, 24.03.1996, *Norris 87411 with Shevock & Barahona, MAUAM 5092, [BVAF71];* California, Mariposa Co., Yosemite N.P., Merced River, Pahono Bridge, 27.10.2008, *Lara, Garilleti & Albertos, MAUAM 5053, [BVAF80];* California, Mariposa Co., Yosemite N.P., Merced River, Pahono Bridge, 27.10.2008, *Lara, Garilleti & Albertos, MAUAM 5054, [BVAF81];* California, Modoc Co., South Warner Wilderness, Emerson Creek campground, 21.08.2005, *Laeger, MAUAM 5049, [BVAF01];* California, Shasta Co., Lassen Nat. Forest, just below Hat Creek Rim, 30.10.2008, *Lara et al., MAUAM 3202, [-];* California, Siskiyou Co., near end of Truck Village Dr. (old Hwy.), 13.02.2004, *Lenz 184, UC1771631, [BVAF70];* Montana, Glacier N.P. (West), Going to the Sun Road, between The Loop Trailhead and Avalanche Campground, 24.08.2015, *Calleja & Vigalondo, MAUAM 5052, [BVAF77];* Montana, Glacier N.P. (West), Going to the Sun Road, between The Loop Trailhead and Avalanche Campground, 24.08.2015, *Calleja & Vigalondo, MAUAM 5052, [BVAF78];* Nevada, Elko Co., Lower Bluster Campsite, 25.08.2002, *Shevock, MAUAM 5050, [BVAF02];* Nevada, Humboldt Co., Humboldt Nat. Forest, Santa Rosa Mts., Buffalo Creek, 29.10.2008, *Lara et al., MAUAM 5056, [-];* Nevada, Toiyabe Nat. Forest, Anchorite Hills, Box Canyon, 28.10.2008, *Lara et al., MAUAM 5055, [-];* Wyoming, Yellowstone N.P., Seven Mile Hole Trail, Yellowstone river, 17.08.2015, *Calleja & Vigalondo, MAUAM 5051, [BVAF76]*.

Lewinskya fastigiata (Bruch ex. Brid) Vigalondo, F.Lara & Garilleti.

Cyprus: Zentrales Troodos-Gebirge, Nordhang des Olymp, Bachtal mit Kiefern-Eichenwald oberhalb Kakopetria, 27.05.2002, *Schäfer-Verwimp 22885, VAL s.n., [BVAF17];* **France:** Rhone-Alps, Hautes-Alpes, Les Écrins, Vallée de la Clarée, Plampinet, 24.07.2013, *Garilleti 2013-21a & Albertos, VAL s.n., [BVAF48];* Embrun, close to the village camping, 07.08.2016, *Lara 1608/01, MAUAM 5057, [-];* **Italy:** Lombardia, Valtellina, between S. Martino and Val di Mello, 19.08.2013, *Lara, MAUAM 3357, [BVAF45];* **Morocco:** Jbel Buhala, Jbel Buhala, Bab-Taza, 16.06.1997, *Albertos et al., MAUAM 2704, [BVAF03];* Ketama, Way up to Jbel Tiridhine, dirty road from Ketama to Tatlaketama, 18.06.1997, *Albertos et al., MAUAM 2705, [BVAF04];* Azrou, 09.11.1989, *MAUAM 2212, [-];* **Romania:** Transilvania, Close to Scoreiu, Transjagara, 24.08.2003, *Lara, MAUAM 5059, [BVAF20];* **Spain:** Burgos, Espinosa de los Monteros, hostel of the village, 11.06.2008, *Garilleti & Lara, VALf 9274, [-];* Cuenca, Tragacete, Cañada Real Conquense, 05.06.2004, *Draper, Medina &*

Pokorny, MAUAM 3222, [-]; Jaen, Sierra de Cazorla, way from Parador de Cazorla to Puerto del Tejo, 20.04.2006, *Garilleti & Albertos, VALf 9325, [-];* Lleida, Sierra Cadí Norte, Barranco Ortedó, 17.07.1998, *Garilleti et al., MAUAM 1664, [BVAF40];* Orense, Viana do Bolo, San Agustín, 07.12.1995, *Albertos et al., MAUAM 1255, [BVAF59];* Orense, *Albertos et al., MAUAM 2219, [BVAF60];* **Turkey:** Artvin, Road between Sarigol and Barhal, NW of Yusufeli, 12.07.2005, *Lara et al., MAUAM 4449, [BV016];* Gümüshane, Road from Kürtün to Tirebolu, 15.07.2005, *Lara et al., MAUAM 5058, [BVAF05]*.

***Lewinskya leptocarpa* (Bruch & Schimp. ex Müll. Hal.) Vigalondo, F.Lara & Garilleti**.

Ethiopia: Amhara, N. Gonder, Simien Mts., Ambaras, Guimbar river, 20.11.2013, *Vigalondo s.n. with Lara & Mazimpaka, MAUAM 5072, [BVAF69];* Amhara, N. Gonder, Simien Mts., below Geech camp, 18.11.2013, *Lara 1311/05 with Mazimpaka & Vigalondo, MAUAM 5065, [BVAF33];* Oromiya, Bale Mts., Harenna Forest, near Rira, 09.11.2013, *Lara 1311/036 with Mazimpaka & Vigalondo, MAUAM 5071, [BVAF68];* Oromiya, Bale Mts., road from Dodola to Dinsho, 06.11.2013, *Lara 1311/71 with Mazimpaka & Vigalondo, MAUAM 5064, [BVAF32]; MAUAM 5070, [-];* **Kenya:** Mt. Kenya, Chogoria route, way from Mintos camp to Meru Bandas, 16.08.2014, *Vigalondo K003-14 et al.,, MAUAM 5062, [BVAF30];* Mt. Kenya, Near Chogoria gate, 16.08.2014, *Lara 1408/24 et al., MAUAM 5069, [BVAF66];* Mt. Kenya, Near Old Moses camp, 13.08.2014, *Garilleti 2014-31 et al., MAUAM 5063, [BVAF31]; MAUAM 5068, [BVAF65];* Mt. Kenya, Sirimon Gate, 12.08.2014, *Calleja s.n. et al., MAUAM 5067, [BVAF64];* **Tanzania:** Mt. Kilimanjaro, Close to Mweca camp, 26.08.2014, *Vigalondo s.n. et al., MAUAM 5066, [BVAF63];* Mt. Kilimanjaro, Machame Route, Shira Cave, 21.08.2014, *Lara 1408/67 et al., MAUAM 5061, [BVAF29];* Ngorongoro C.A., Nainokanoka village, 28.08.2014, *Lara 1408/40 et al., MAUAM 5060, [BVAF28]*.

***Lewinskya pacifica* Vigalondo, F.Lara & Garilleti.**

Canada: British Columbia, Vancouver Island, Victoria, university gardens of Mistra Val, 05.12.2001, *Lara, MAUAM 5076, [-];MAUAM 5077, [-];* **USA:** California, Lake Co., south shore of Clear Lake at Corinthian Bay County Park., 21.12.1984, *Norris 71798, UC - DHN71798 , [-];* Oregon, Washington Co., Hillsboro, Confort Inn Parking, 28.07.2011, *Lara, Garilleti & Albertos, MAUAM 5075, [BVAF79];* Washington, Skamania Co., Columbia river, Beacon Rock State Park, 27.07.2011, *Lara et al., MAUAM 5073, [BVAF50]; MAUAM 5078, [-];* Washington, Whatcom Co., Bellingham, Sunset Square shopping centre, 20.07.2011, *Lara & Garilleti, MAUAM 5074, [BVAF72]*.

Lewinskya pseudoaffinis Vigalondo, F.Lara & Garilleti.

Canada: British Columbia, Central Coast Regional District, Bella Coola, dirty road to Clynton Fall Creek, 07.08.2011, *Lara et al., MAUAM 5084, [BVAF13]; MAUAM 5086, [-]*; British Columbia, Squamish-lillooet Regional District, E of Pemberton, Lillooet Lake, 05.08.2011, *Lara & Garilleti, MAUAM 5082, [BVAF74]; MAUAM 5083, [BVAF75];* British Columbia, Squamish-lillooet Regional District, Lillooet Lake, 05.08.2011, *Lara & Garilleti, MAUAM 5085, [-];* **USA:** California, Shasta Co., Along Soda Creek, south of Dunsmuir, 10.05.2002, *Norris et al., MAUAM 4447, [BVAF14]*; Washington, Whatcom Co., Okanoga Nat. Forest, Canyon Creek Trail, 22.07.2011, *Lara et al., MAUAM 5087, [-]; MAUAM 5080, [BVAF51];* Washington, Whatcom Co., Ross Lake, near the Canadian border, Obelisk Trail, 20.07.2011, *Lara & Garilleti, MAUAM 5081, [BVAF73]*.

Lewinskya scissa Vigalondo, F.Lara & Garilleti.

Spain: Islas Canarias. Gran Canaria, Las Cumbres, 11.11.2016, *Calleja 03-2016, MAUAM 5090, [-];* Gran Canaria, Pico de Las Nieves, 22.04.2004, *Medina, MAUAM 5089, [BVAF83];* Gran Canaria, road GC-21, near view point of Pinos de Galdar, 23.06.2012, *Vigalondo & Calleja, MAUAM 5088, [BVAF56];* Gran Canaria, Valleseco, Madrelagua, 12.11.2016, *Calleja 10-2016, MAUAM 5091, [-]*.

Supplementary Material

Table 3.4.S1. Marginal likelihood (MLE) and Bayes factor (BF) values for alternative clocks and models tested in BEAST. The best model is marked in bold.

		Path Sampling		Stepping-Stone	
		ln(MLE)	2*ln*(BF)	*ln*(MLE)	2*ln*(BF)
Uncorrelated Lognormal Strict Consensus	**Birth-death**	**-7136,22**	**0,00**	**-7136,15**	**0,00**
	Yule	-7154,83	37,20	-7155,27	38,24
	Birth-death	-7144,07	15,69	-7143,97	15,64
	Yule	-7166,98	61,52	-7167,83	63,36

Table 3.4.S2. Qualitative morphological characters evaluated for the *Lewinskya affinis* complex. (*) Multistate characters transformed to binary characters (no/yes = 0/1) for multivariate statistical analyses. Binary character states are separated by " / ". ID number correspond to the number of the character as included in the data set for morphometric analyses. ([1]) Calyptra hairiness refers to the number of hairs viewed in one side of the calyptra. ([2]) Capsule characters refers to dry and empty capsules.

Character	ID	Character state
Vegetative leaves		
Shape *	1	Narrowly lanceolate
	2	Lanceolate
	3	Broadly lanceolate
	4	Oval-lanceolate
Apex shape *	5	Blunt
	6	Acute
	7	Apiculate
	8	Acuminate
Apex symmetry	9	Asymmetric / Symmetric
Margin	10	Recurved / Revolute
Perichaetial leaves		
Shape *	11	Lanceolate
	12	Broadly lanceolate
	13	Oval-lanceolate
Apex *	14	Blunt
	15	Acute
	16	Apiculate
	17	Acuminate
Apex symmetry	18	Asymmetric / Symmetric
Margin	19	Recurved / Revolute
Calyptra		
Calyptra hairiness * [(1)]	20	None or very few and inconspicuous short hairs
	21	Few (<10)
	22	Abundant (10-20)
	23	Very abundant (>20)
Calyptra hairs length	24	Short (less than 1/3 of calyptra) / Long (more than 1/3 of calyptra)
Calyptra hairs position	25	Accumulated in the upper third of the calyptra / Scattered along the calyptra
Capsule [(2)]		
Position respect to perichaetial leaves *	26	Inmersed
	27	Shortly emergent
	28	Hemiemergent
	29	Longly emergent
	30	Shortly exerted

Table 3.4.S2. Continuation.

Character	ID	Character state
Shape *	31	Cylindrical not constricted, nor contracted
	32	Cylindrical contracted below mouth
	33	Urceolate, constricted in the upper half
	34	Oval-cylindric
Width	35	Relatively broad (<3:1) / Relatively narrow (>4:1)
Capsule furrows *	36	Slightly furrowed
	37	Moderately furrowed
	38	Deeply furrowed
Exothecial bands		
Differentiation at capsule mouth	39	Almost reaching the mouth, separate by 1-5 thin oblate cells, sometimes forming a clear ring interrupted by the exothecial band / Clearly not reaching the mouth, separate by a clear ring of short cells not interrupted by the exothecial band
Cell differentiation respect to whole urn *	40	< 1/3 of the urn
	41	1/3 - 2/3 of the urn
	42	2/3 to the whole urn
Peristome		
Exostome general aspect	43	Remaining in pairs with age / Easily splitting after the detachment of the lid
Exostome teeth colour	44	Orange / Whitish-yellowish or brownish
Exostome teeth aspect	45	Trabeculate at the apex, cancellate and/or fenestrate / Not trabeculate/cancellate at the apex
Exostome outter layer ornamentation (OPL) *	46	Predominantly lines, striate or vermicular
	47	Mixed short lines and papillae
	48	Papillose
Endostome number of segments	49	8 / 8+n (intermediary or remains)
Endostome number of cells per segment	50	Uniseriate or (and) partially biseriate / All partially biseriate or biseriate
Endostome inner layer ornamentation (IPL)	51	Strongly trabeculate / Not trabeculate or slightly trabeculate
Spore ornamentation	52	Verrucose or papillose with thin or thick stripes / Papillose without stripes

Figure 3.4.S1. Maximum-clade-credibility tree from the relaxed-clock analysis of four loci in the *Lewinskya affinis* complex using a Birth-death model in BEAST on the reduced dataset. The different colored bars on top indicate the estimated entities from the ML multiple-threshold and Bayesian GMYC models (mGMYC and bGMYC), as well as the lineages revealed by the BI and ML analyses, and the best model of BFD approach.

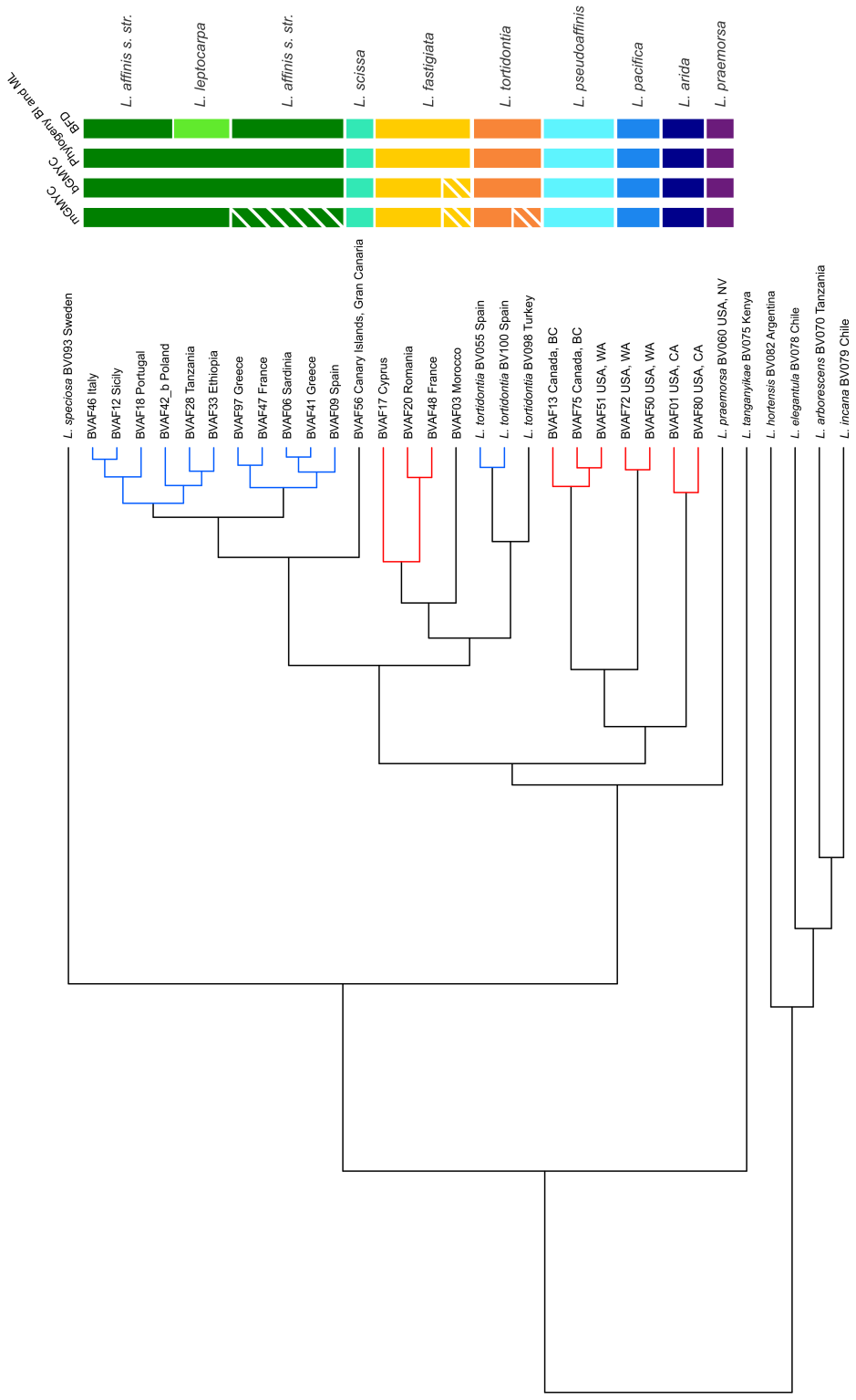

Figure 3.4.S2. Biplots of the PCA analyses of quantitative traits, representing the first three principal components. Samples are colored following the best hypothesis model obtained in BFD analysis. Circle = Old World, triangle = New World. The larger circles and triangles correspond to the centroid of each group.

Table 3.4.S3. Correlations between quantitative variables and principal components from the PCA analysis of *Lewinskya affinis s.l.* In bold, variables with the highest coefficients for each component. Character abbreviation as in Figure 3.4.3A.

Character	Character abreviation	PC1	PC2	PC3
Gametophyte				
Plant height	High	-0,203	-0,047	-0,120
Perichaetial leaf length	PeLL	**-0,323**	0,003	0,038
Perichaetial leaf width	PeLW	**-0,309**	0,193	-0,176
Perichaetial leaf acumen length	PeLAcL	-0,180	**-0,304**	0,196
Upper leaf length	UpLL	**-0,359**	-0,001	0,124
Upper leaf width	UpLW	-0,296	0,181	-0,231
Upper leaf acumen length	UpLAcL	-0,190	**-0,314**	0,286
Leaf costa width at base	NerWB	-0,147	**0,320**	**-0,348**
Leaf costa width at central lamina	NerWM	-0,146	**0,381**	-0,231
Leaf lamina cell length	CelLamL	-0,207	0,094	0,285
Leaf lamina cell width	CelLamW	-0,271	0,074	0,176
Sporophyte				
Vaginula length	VagL	-0,270	0,093	0,040
Seta length	SetaL	-0,220	-0,123	0,232
Capsule length	CapL	-0,265	0,179	0,202
Capsule neck length	NeckL	-0,090	0,220	0,279
Exotecial band width	ExBW	0,166	**0,395**	0,215
Exotecial band cell length	ExBCelL	0,109	0,241	**0,382**
Exotecial band cell width	ExBCelW	0,156	**0,302**	0,287
Spore diameter	SporeL	-0,228	-0,256	-0,170

Figure 3.4.S3. Biplots of the PCA analyses of qualitative traits, representing the first three principal components. Samples are colored following the best hypothesis model obtained in BFD analysis. Circle = Old World, triangle = New World. The larger circles and triangles correspond to the centroid of each group.

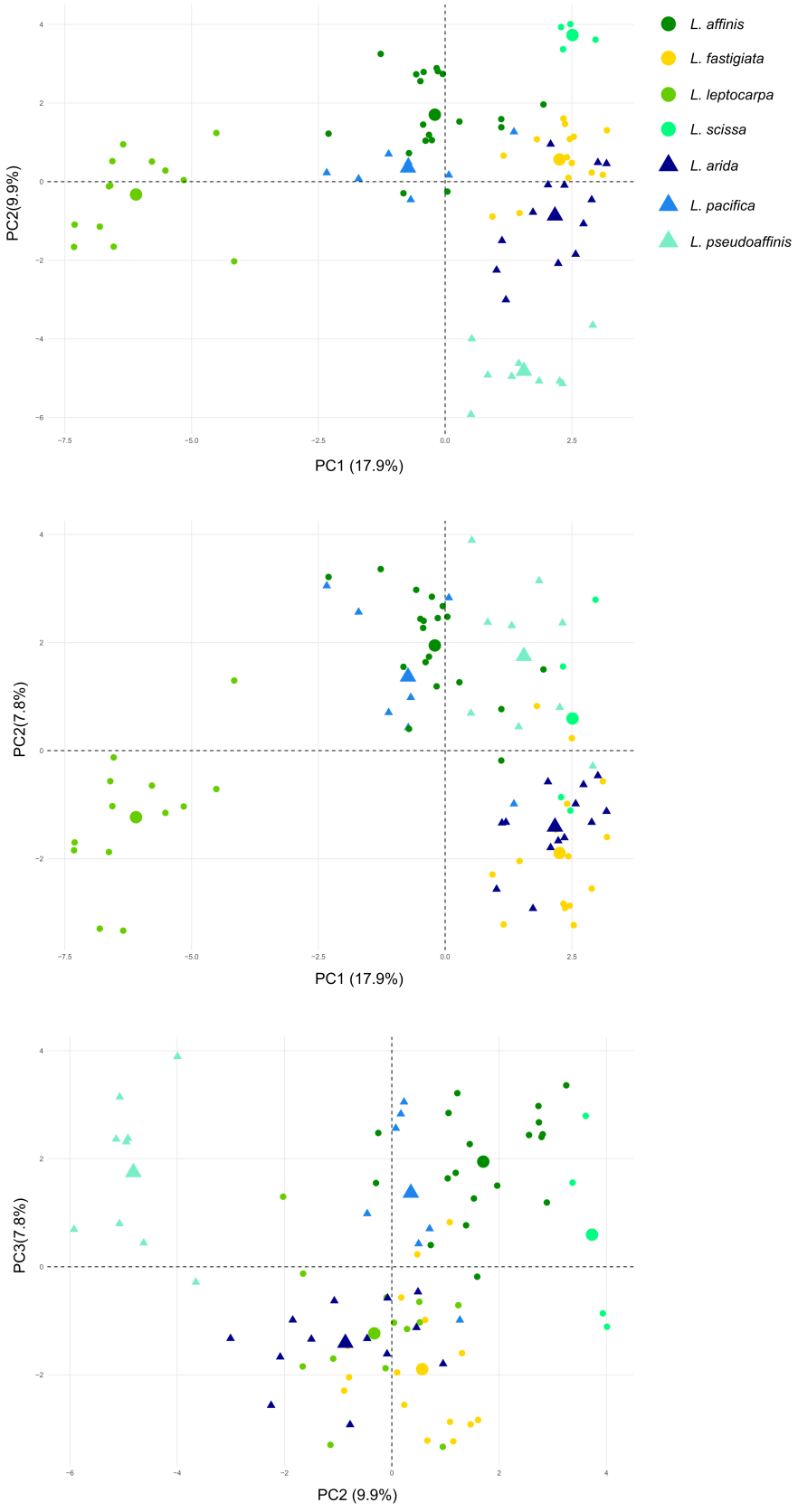

Table 3.4.S4. Correlations between qualitative variables and principal components from the PCA analysis of *Lewinskya affinis s.l.* In bold, variables with the highest coefficients for each component. Characters ID are explained in table 3.4.S2.

Character ID	PC1	PC2	PC3	Character ID	PC1	PC2	PC3
1	0,014	-0,098	-0,009	27	0,000	0,076	-0,107
2	0,087	-0,046	0,263	28	-0,128	-0,112	-0,047
3	-0,022	0,099	-0,279	29	0,086	-0,156	0,090
4	-0,168	-0,015	-0,107	31	-0,134	0,093	0,163
5	-0,017	0,010	0,095	32	0,030	0,126	0,135
6	-0,006	0,252	0,002	33	0,227	-0,011	-0,270
7	-0,178	-0,065	-0,177	34	-0,174	-0,051	0,088
8	0,092	-0,267	0,065	35	0,188	-0,156	-0,126
9	-0,015	0,067	0,038	36	-0,144	-0,009	-0,080
10	-0,034	0,044	**-0,324**	37	-0,146	-0,082	**0,313**
11	0,010	0,006	0,210	38	0,186	0,096	**-0,280**
12	0,221	0,043	-0,079	39	**-0,294**	-0,028	-0,135
13	**-0,276**	-0,047	-0,101	40	**-0,266**	-0,038	-0,130
14	-0,018	0,006	0,101	41	-0,108	0,074	0,032
15	0,156	0,164	-0,154	42	0,259	-0,019	0,060
16	-0,206	0,116	0,103	43	0,016	0,165	0,034
17	0,080	**-0,300**	0,065	44	-0,230	0,045	-0,035
18	-0,069	0,128	-0,134	45	-0,211	0,187	0,161
19	0,007	-0,058	-0,130	46	-0,046	-0,190	-0,146
20	0,038	0,077	-0,053	47	0,039	0,209	0,106
21	0,121	**0,290**	0,083	48	0,022	0,019	0,045
22	-0,193	-0,092	-0,172	49	0,056	0,114	0,051
23	0,036	**-0,319**	0,163	50	0,102	0,006	0,031
24	-0,130	-0,223	0,058	51	0,109	0,195	0,056
25	0,149	-0,186	0,054	52	-0,144	0,213	0,126
26	0,067	0,185	0,062				

Figure 3.4.S4. Maximum clade credibility trees from Bayesian species tree reconstructions of each of the nine hypotheses tested (H1-H9, Table 3.4.2). Numbers on branches represent posterior probabilities (PP). Green lines represent the lineages of the *Lewinskya affinis* complex with high support for each tree (PP > 0.80).

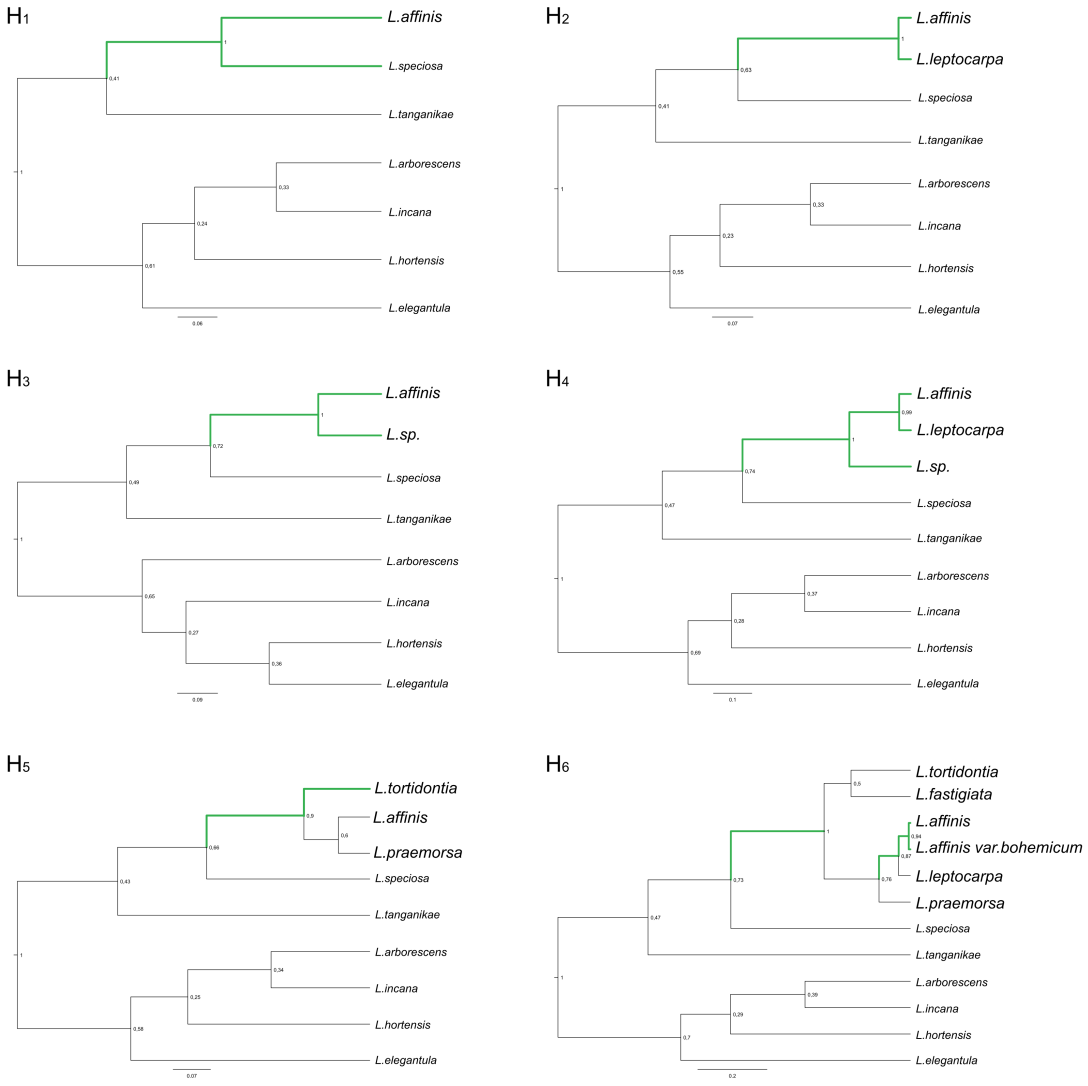

Figure 3.4.S4. Continuation**.**

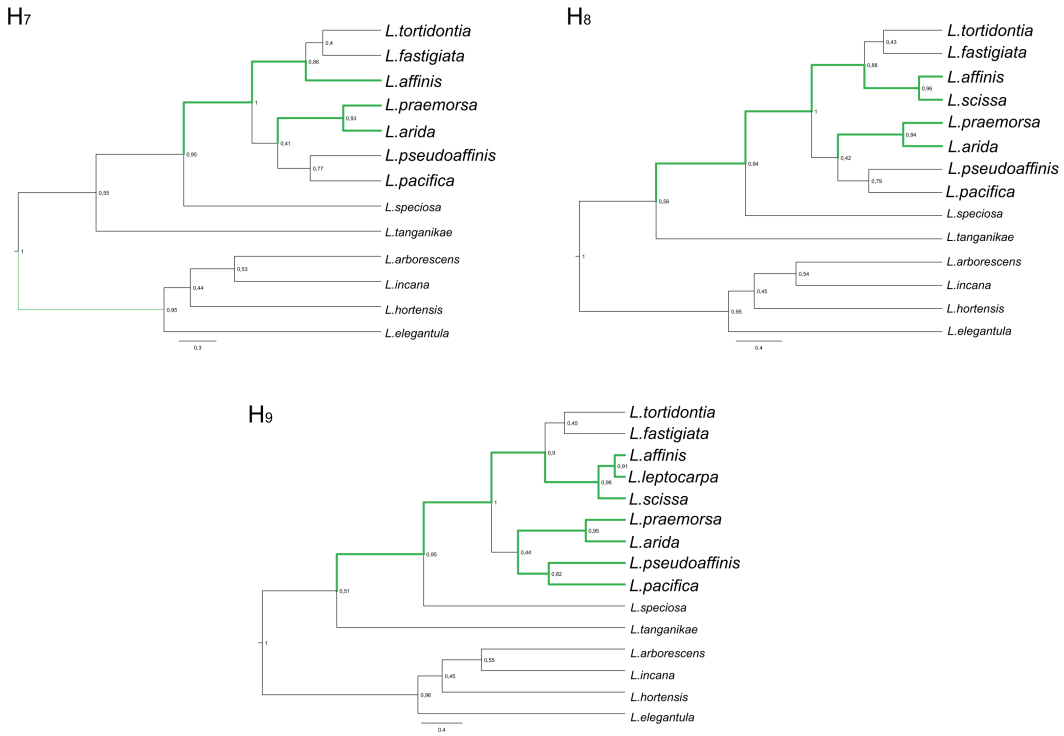

Figure 3.4.S5. Discriminant function analysis (DFA) of *Lewinskya affinis s.l.* for the mixed qualitative and quantitative traits representing the first three axis. Samples are colored following the best hypothesis model for the *Lewinksya affinis* complex obtained in BFD analyses. Circle = Old World, triangle = New World.

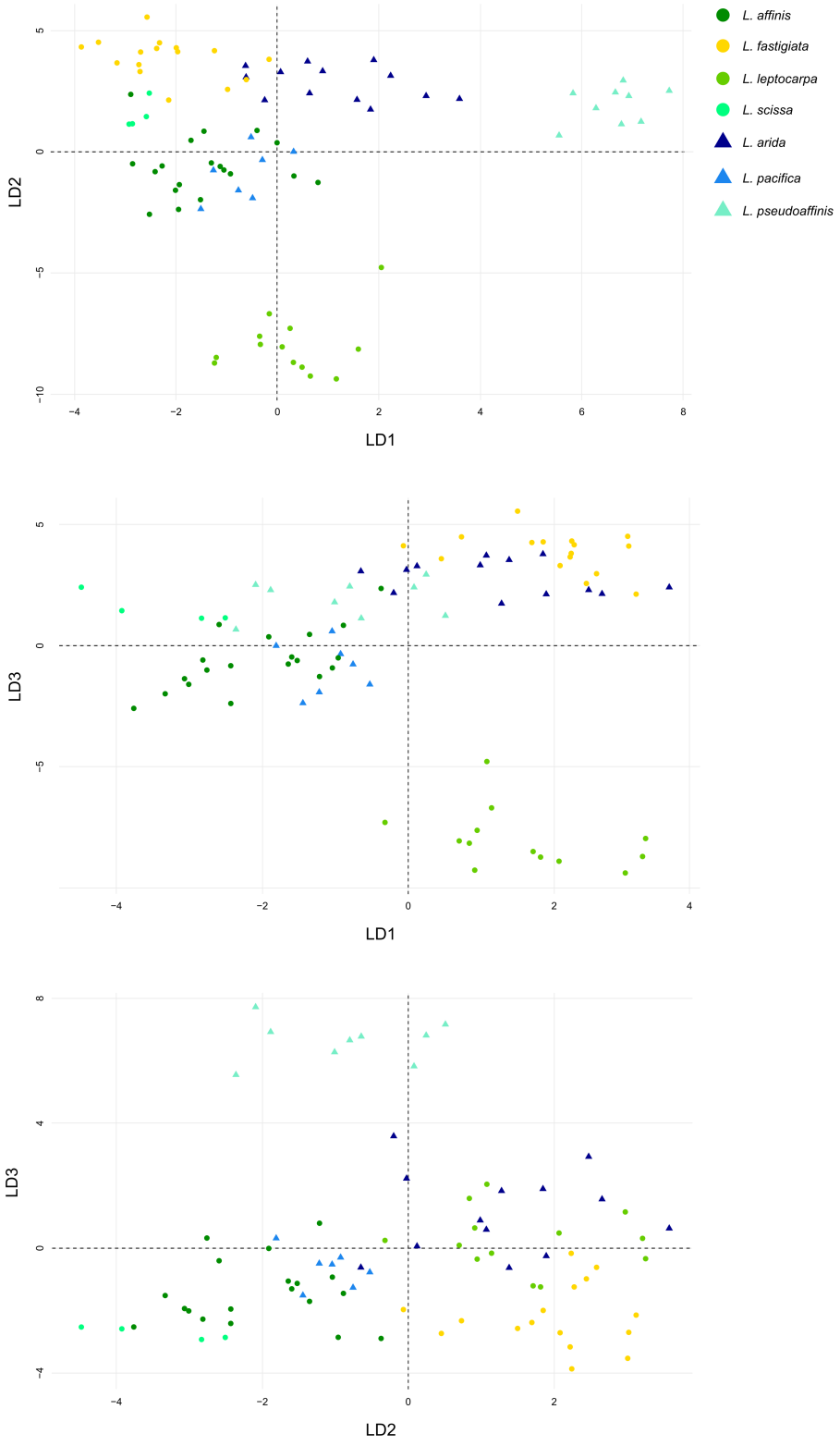

Figure 3.4.S6. Beanplots of the studied quantitative variables for the seven obtained species within *Lewinskya affinis s.l.* Individual observations are represented by small horizontal lines (in the case of multiple observations with the same values, the corresponding number of lines were merged), mean per group is shown by a bold long line and the mean for all data by a dotted line. Estimated density of the data distribution is displayed by the density colored shape (for details see Kampstra, 2008). Stars indicate ANOVA significance values: *** 0.001, ** 0.01, * 0.05.

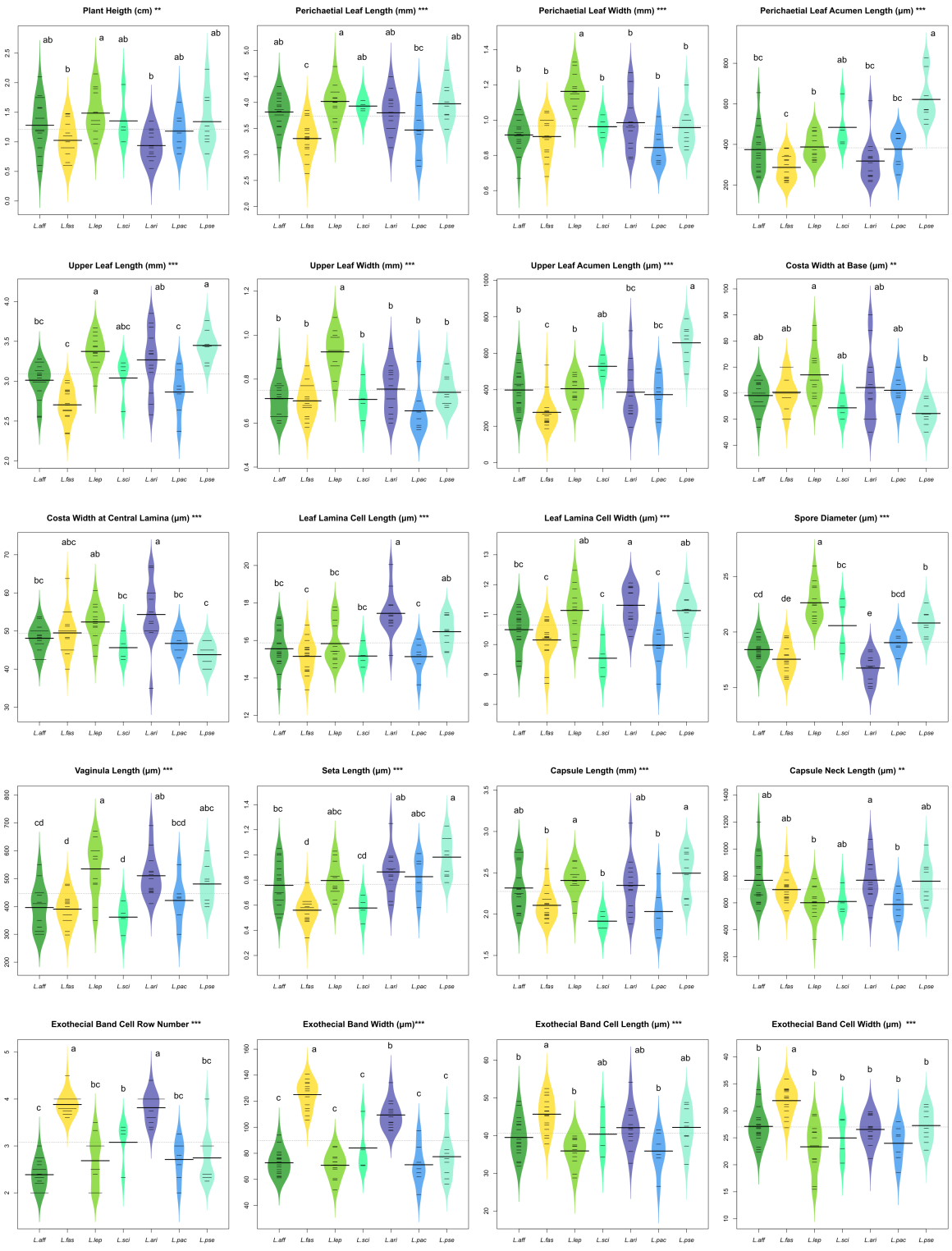

Table 3.4.S5. Selection of specimens used for molecular analyses, including voucher information. According to the guidelines, GenBank accession numbers will be provided after the manuscript is accepted.

				Genebank accesion number			
Taxa	**DNA ID**	**Country**	**Voucher**	***rps* 4**	***rpl* 32-*trn* L$^{(UAG)}$**	***EST*-115**	***EST*-317**
Lewinskya affinis	BVAF06	Italy, Sardinia	MAUAM 3343	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF07	Netherlands, Groningen	MAUAM 3348	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF08	Slovenia, Jesenice	MAUAM 3350	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF09	Spain, Segovia	MAUAM 1958	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF10	Czech Republic, Moravia	MAUAM 3355	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF11	Germany, Saxony Anhalt	MAUAM 3356	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF12	Italy, Sicily	MAUAM 3354	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BV015	Spain, Jaen	MAUAM 4448	KT862306	*XX000000*	--	*XX000000*
L. affinis	BVAF18	Portugal, Algarve	MAUAM 5047	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF19	Poland, Mazovia	MAUAM-3353	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF22	Germany, Bavaria	MAUAM-3351	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF27	Switzerland, Valais	VAL s.n.	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF35	Spain, Ciudad Real	MAUAM 1675	*XX000000*	*XX000000*	--	--
L. affinis	BVAF36	United Kingdom, Lake District	MAUAM 2912	*XX000000*	–	--	--
L. affinis	BVAF37	Portugal, Tras-os-Montes	MAUAM 2894	*XX000000*	–	--	--
L. affinis	BVAF38	Spain, La Rioja	VAL s.n.	*XX000000*	*XX000000*	*XX000000*	--
L. affinis	BVAF41	Greece, Ipiros	MAUAM 3345	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF42	Poland, Warmia-Masuria	MAUAM 3346	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF43	Germany, Saxony Anhalt	MAUAM 3347	*XX000000*	–	*XX000000*	*XX000000*
L. affinis	BVAF44	Belgium, Wallonia	MAUAM 3349	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF46	Italy, Piemonte	VAL s.n.	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF47	France, Languedoc-Roussillon	VAL s.n.	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF57	Czech Republic, Bohemia	OSTR s.n.	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. affinis	BVAF58	Spain, Lugo	MAUAM 1275	*XX000000*	--	--	--
L. affinis	BVAF61	Spain, Zamora	MAUAM 1245	*XX000000*	*XX000000*	*XX000000*	--
L. affinis	BVAF62	Spain, Lugo	MAUAM 1227	*XX000000*	*XX000000*	--	--

Table 3.4.S5. Continuation.

Taxa	DNA ID	Country	Voucher	Genebank accesion number			
				rps 4	*rpl* 32-*trn* L$^{(UAG)}$	*EST*-115	*EST*-317
L. affinis	BVAF97	Greece, Eastern Makedonia	Blockeel 36/416	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. arida	BVAF01	USA, California	MAUAM 5049	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. arida	BVAF02	USA, Nevada	MAUAM 5050	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. arida	BVAF52	USA, California	MAUAM 5048	*XX000000*	*XX000000*	*XX000000*	--
L. arida	BVAF70	USA, California	UC1771631	*XX000000*	--	--	--
L. arida	BVAF71	USA, California	MAUAM 5092	*XX000000*	--	--	--
L. arida	BVAF76	USA, Wyoming	MAUAM 5051	*XX000000*	*XX000000*	--	--
L. arida	BVAF77	USA, Montana	MAUAM 5052	*XX000000*	--	*XX000000*	*XX000000*
L. arida	BVAF78	USA, Montana	MAUAM 5052	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. arida	BVAF80	USA, California	MAUAM 5053	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. arida	BVAF81	USA, California	MAUAM 5054	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. fastigiata	BVAF03	Morocco, Chefchaouen	MAUAM 2704	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. fastigiata	BVAF04	Morocco, Taza-Alhucemas-Taunat	MAUAM 2705	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. fastigiata	BVAF05	Turkey, Gümüshane	MAUAM 5058	*XX000000*	*XX000000*	*XX000000*	--
L. fastigiata	BV016	Turkey, Artvin	MAUAM 4449	KT862307	*XX000000*	*XX000000*	--
L. fastigiata	BVAF17	Cyprus	VAL s.n.	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. fastigiata	BVAF20	Romania, Transylvania	MAUAM 5059	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. fastigiata	BVAF40	Spain, Lleida	MAUAM 1664	*XX000000*	--	--	--
L. fastigiata	BVAF48	France, Rhone-Alps	VAL s.n.	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. fastigiata	BVAF59	Spain, Orense	MAUAM 1255	*XX000000*	*XX000000*	--	--
L. fastigiata	BVAF60	Spain, Orense	MAUAM 2219	*XX000000*	*XX000000*	--	--
L. leptocarpa	BVAF28	Tanzania, Ngorongoro	MAUAM 5060	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. leptocarpa	BVAF29	Tanzania, Mt. Kilimanjaro	MAUAM 5061	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. leptocarpa	BVAF30	Kenya, Mt. Kenya	MAUAM 5062	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. leptocarpa	BVAF31	Kenya, Mt. Kenya	MAUAM 5063	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. leptocarpa	BVAF32	Ethiopia, Oromiya	MAUAM 5064	*XX000000*	*XX000000*	*XX000000*	--

Table 3.4.S5. Continuation.

Taxa	DNA ID	Country	Voucher	Genebank accesion number			
				rps 4	*rpl* 32-*trn* L$^{(UAG)}$	*EST*-115	*EST*-317
L. leptocarpa	BVAF33	Ethiopia, Amhara	MAUAM 5065	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. leptocarpa	BVAF63	Tanzania, Mt. Kilimanjaro	MAUAM 5066	*XX000000*	*XX000000*	*XX000000*	--
L. leptocarpa	BVAF64	Kenya, Mt. Kenya	MAUAM 5067	*XX000000*	*XX000000*	*XX000000*	--
L. leptocarpa	BVAF65	Kenya, Mt. Kenya	MAUAM 5068	*XX000000*	*XX000000*	--	--
L. leptocarpa	BVAF66	Kenya, Mt. Kenya	MAUAM 5069	*XX000000*	*XX000000*	--	*XX000000*
L. leptocarpa	BVAF68	Ethiopia, Oromiya	MAUAM 5071	*XX000000*	*XX000000*	--	--
L. leptocarpa	BVAF69	Ethiopia, Amhara	MAUAM 5072	*XX000000*	*XX000000*	*XX000000*	--
L. pacifica	BVAF50	USA, Washington	MAUAM 5073	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pacifica	BVAF72	USA, Washington	MAUAM 5074	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pacifica	BVAF79	USA, Oregon	MAUAM 5075	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pseudoaffinis	BVAF13	Canada, British Columbia	MAUAM 5084	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pseudoaffinis	BVAF14	USA, California	MAUAM 4447	*XX000000*	*XX000000*	--	--
L. pseudoaffinis	BVAF51	USA, Washington	MAUAM 5080	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pseudoaffinis	BVAF73	USA, Washington	MAUAM 5081	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pseudoaffinis	BVAF74	Canada, British Columbia	MAUAM 5082	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pseudoaffinis	BVAF75	Canada, British Columbia	MAUAM 5083	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. scissa	BVAF56	Spain, Canary Is., Gran Canaria	MAUAM 5088	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. scissa	BVAF83	Spain, Canary Is., Gran Canaria	MAUAM 5089	*XX000000*	--	--	--
L. acuminata	BV002	USA, California	MAUAM 3317	KT862303	--	*XX000000*	*XX000000*
L. acuminata	BV006	France, Corse	MAUAM 3164	KT862293	*XX000000*	*XX000000*	*XX000000*
L. arborescens	BV070	Tanzania, Mt. Kilimanjaro	MAUAM 4590	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. armatum	BV071	South Africa, Western Cape	MAUAM 4566	*XX000000*	*XX000000*	*XX000000*	--
L. bolanderi	BV059	USA, California	MAUAM 4572	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. breviseta	BV095	Spain, Málaga	MAUAM 4610	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. brotheri	BV077	Chile, Provincia Capitán Prat	MAUAM 4583	*XX000000*	--	--	--
L. elegantula	BV078	Chile, Magallanes	MAUAM 4575	*XX000000*	*XX000000*	*XX000000*	*XX000000*

Table 3.4.S5. Continuation.

Taxa	DNA ID	Country	Voucher	*rps*4	*rpl*32-*trn*L$^{(UAG)}$	*EST*-115	*EST*-317
L. firma	BV072	Tanzania, Ngorongoro	MAUAM 4591	*XX000000*	*XX000000*	--	--
L. galiciae	BV073	Ethiopia, Amhara	MAUAM 4592	*XX000000*	*XX000000*	*XX000000*	--
L. holzingeri	BV061	USA, Washington	MAUAM 4571	*XX000000*	*XX000000*	--	--
L. hookeri	BV086	Nepal, Rasuwa District	MAUAM 4577	*XX000000*	--	--	--
L. hortensis	BV082	Argentina, Neuquén	MAUAM 2982	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. hortoniae	BV057	Mexico, Mexico State	MAUAM 4584	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. iberica	BV056	Spain, Ávila	MAUAM 4567	*XX000000*	*XX000000*	*XX000000*	--
L. incana	BV079	Chile, Provincia Capitán Prat	MAUAM 4576	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. incurvomarginata	BV074	South Africa	MAUAM 4569	*XX000000*	*XX000000*	*XX000000*	--
L. laevigata	BV034	Spain, Madrid	MAUAM 4461	KT862308	*XX000000*	*XX000000*	*XX000000*
L. ludificans	BV080	Chile, Provincia Capitán Prat	MAUAM 4582	*XX000000*	*XX000000*	*XX000000*	--
L. mandonii	BV081	Bolivia, La Paz	MAUAM 4573	*XX000000*	*XX000000*	--	--
L. papilosa	BV062	Canada, British Columbia	MAUAM 4585	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. praemorsa	BV060	USA, Nevada	MAUAM 4570	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. pycnophylla	BV076	Mexico, Mexico State	MAUAM 4580	*XX000000*	*XX000000*	--	--
L. pylaisii	BV066	Norway, Svalbard and Jan Mayen	MAUAM 4581	*XX000000*	*XX000000*	--	*XX000000*
L. rupestris	BV067	Switzerland, Valais	MAUAM 4586	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. sainsburyi	BV084	New Zeland, South Island	MAUAM 2103	*XX000000*	*XX000000*	--	--
L. shawii	BV068	Greece, Ipiros	MAUAM 4587	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. sordida	BV063	USA, Alaska	MAUAM 4588	*XX000000*	*XX000000*	--	--
L. speciosa	BV022	Turkey, Artvin	MAUAM 3061	KT862313	*XX000000*	--	*XX000000*
L. speciosa var. speciosa	BV093	Sweden, Uppland	MAUAM 2818	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. spjutii	BV058	USA, California	MAUAM 4589	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. striata	BV013	Turkey, Antalya	MAUAM 4446	KT862316	--	*XX000000*	--
L. striata	BV069	Spain, Cáceres	MAUAM 4579	*XX000000*	*XX000000*	*XX000000*	--
L. tanganyikae	BV075	Kenya, Mt. Kenya	MAUAM 4593	*XX000000*	*XX000000*	*XX000000*	*XX000000*

Table 3.4.S5. Continuation**.**

Taxa	DNA ID	Country	Voucher	Genebank accesion number			
				rps **4**	*rpl* **32-** *trn* **L**$^{(UAG)}$	***EST*-115**	***EST*-317**
L. tasmanica	BV085	New Zeland, South Island	MAUAM 2162	*XX000000*	*XX000000*	--	--
L. tortidontia	BV055	Spain, Ávila	MAUAM 4434	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. tortidontia	BV098	Turkey, Antalya	MAUAM 4603	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. tortidontia	BV099	Morocco, Azrou	M-3220506	*XX000000*	*XX000000*	--	--
L. tortidontia	BV100	Spain, Málaga	MAUAM 4606	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. tortidontia	BV101	Greece	MAUAM 2068	*XX000000*	*XX000000*	*XX000000*	*XX000000*
L. vladikavkana	BV087	Turkey, Trabzon	MAUAM 3075	*XX000000*	*XX000000*	--	--
Macrocoma lycopodioides	BV024	South Africa, Western Cape	MAUAM 2953	KT862288	*XX000000*	*XX000000*	*XX000000*
Pulvigera lyelii	BV065	Spain, Málaga	MAUAM 4578	*XX000000*	*XX000000*	*XX000000*	*XX000000*
Zygodon pentastichus	ID207	Argentina, Córdoba	MAUAM 2910	KT862290	--	--	--
Z. viridissimus	ID208	United Kingdom, Lake District	MAUAM 2981	KT862289	*XX000000*	--	--

Chapter

4

Discussion

Species delimitation in bryophytes

The correct delimitation of species boundaries is a key factor to understand the actual biodiversity of the planet, which is important in different fields such as biodiversity assessment, ecology, and conservation. Within this thesis, three different bryophyte species of the tribe Orthotricheae have been studied and the results support the need of performing accurate species delimitations for biogeographic studies of bryophytes, especially among taxa that display wide and disjunct distributions.

The results of the different studies here addressed evidence that there is still a considerable gap of knowledge on bryophyte diversity, both regarding the number of species and the understanding of the species distribution ranges. This is reflected in chapter 3.4 with the discovery of several species within *Lewinskya affinis s.l.*, and in chapters 3.1 and 3.3 with the finding of new and disjunct populations of both *Lewinskya acuminata* and *Orthotrichum shevockii*. These results also add evidences to the fact that the knowledge of bryophyte floras is still less well known than that of angiosperms, independently of the region of the world considered, as evidenced in many recent studies (e.g. Dirkse *et al.*, 2016; Jiménez *et al.*, 2016, 2017; Kiebacher & Lüth, 2016; Bastos & Schäfer-Verwimp, 2017; Sim-Sim *et al.*, 2017).

The underestimation of bryophytes diversity has been related in several occasions to the existence of cryptic species (e.g. Shaw *et al.*, 2008; Heinrichs *et al.*, 2011; Carter, 2012; Buczkowska *et al.*, 2016). This is also the case here, since within *Lewinskya affinis* s.l. six hidden species, including four new to Science, have been discovered. Because of their similarity and close phylogenetic relation all these taxa must be considered as sibling species However, a detailed morphological study has demonstrated the existence of differential traits that allow their safe, although not always easy, discrimination. The classical broad species concept currently prevailing in classifications of bryophytes, together with the overlooking or misinterpretation of some significant morphological characters, have hampered the assessment of the actual diversity in this group, as also has been shown in others studies based on integrative approaches (e.g. Renner *et al.*, 2013, 2017; Aranda *et al.*, 2014; Heinrichs *et al.*, 2015).

Nowadays, the importance of integrating different sources of information to separate species is commonly accepted. However, there is still a considerable number of studies only based on molecular data. In this context, the study of *Lewinskya affinis* reinforces the idea that the lack of molecular evidence does not provide a definitive proof to determine that two or more entities belong to a single species, but rather just fails to provide positive evidence that they are different species (Vanderpoorten & Shaw, 2010). In chapter 3.4 the different molecular methods employed fail to discriminate between *L. leptocarpa* and *L. affinis s.str.*, while morphological analyses clearly differentiate both species, and both are geographically isolated. One reason for this could be that the molecular markers used are inappropriate to differentiate species because of little variation, or that they do not codify for the morphological differences seen among individuals of both species. However other species of the *L. affinis* group, including some morphologically very similar, have been clearly discriminated with the same loci. This fact might point to a recent divergence of *L. leptocarpa* and *L. affinis*, which needs further investigation.

The study of the *Lewinskya affinis* complex also shows that in cases where morphological characters are initially thought to be very variable or overlap, the use of molecular analyses together with a morphological re-evaluation of these characters and other secondary ones, can led to the segregation of different species (e.g. Renner *et al.*, 2013; Draper *et al.*, 2015). In our case, these methods have provided evidences for the description of three new species for western North America and one more for Macaronesia, and for the reinstatement of two previously synonymized species, in line with other similar studies (e.g. Renner *et al.*, 2013; Heinrichs *et al.*, 2015; Caparrós *et al.*, 2016). On the contrary, combining molecular and morphological data can also support the synonymization of different taxa (e.g. Aranda *et al.*, 2014), as concluded in chapter 3.3 with respect to *Orthotrichum kellmanii* and *O. shevockii*. In this case, the wide morphological variation of one species, *O. shevockii*, has been overlooked, and this lead to the description of two different taxa, whereas all populations actually belonged to only one species. Thereby, this thesis supports that for accurately delimiting species boundaries, it is necessary to perform precise and detailed morphological analyses including appropriate statistical methods, and integrate them with molecular data, as well as with other kind of evidences like geography or ecology, which is particularly necessary in taxonomically complex organisms like many bryophytes.

Biogeography. Do mosses really exhibit larger distribution ranges than angiosperms?

The biogeographic disjunction that concerns western Europe and western North America is one of the most common among bryophytes (Schofield, 1988), and particularly within the genera *Orthotrichum* and *Lewinskya* (Lewinsky, 1993; Lara *et al.*, 2016). The three study cases treated in this thesis involved, a priori, species with such a disjunct distribution pattern, although here it is preferred to give a broader geographic scope to the areas involved, thus referring to them as western Palearctic (Europe, Mediterranean Basin and Macaronesia) and western Nearctic (western North America). In the cases of *L. acuminata* and *L. affinis s. l.*, the disjunction also involves East Africa (Paleotropical region) as a third continental area, a pattern rarely studied before for mosses. Meanwhile, *O. shevockii* also displays a western Paleartic – western Nearctic disjunction, but it is only restricted to the Californian region and Macaronesia, which implies an infrequent biogeographic pattern (Grehan, 2017).

The results of the three studied cases partially agree with the view that bryophyte species exhibit large, trans-oceanic distribution ranges due to their long-distance dispersal capacities. In fact, this view matches the conclusions reached for the distribution pattern of *Lewinskya acuminata* and *Orthotrichum shevockii*. However, the western Palearctic - western Nearctic – East Africa disjunction is discarded at species level in the case of *L. affinis*, since it has been revealed as a complex of several species, all of them restricted to one side of the disjunction among the three areas involved. This latter situation of *L. affinis* recalls that previously described for *O. tenellum* (Medina *et al.*, 2013), which only included western Palearctic endemics or western Nearctic ones. In the case of *O. consimile*, the disjunct distribution of the species was also discarded (Medina R. *et al.*, 2012). It was concluded that this species only occurs in western North America, as well as the two new species described in that work, although in this case one species, *O. columbicum*, showed a trans-Atlantic distribution. Also *O. pulchellum* Brunt. was confirmed to have a western Palearctic - western Nearctic disjunct distribution (Medina R. *et al.*, 2012). Furthermore, these two opposite situations, truly disjunct distributions vs. species vicariance, have also been reported for other widespread bryophytes showing other different disjunct ranges (e.g. Lewis *et al.*, 2014; Patiño *et al.*, 2016, and Hedenäs *et al.*, 2014; Heinrichs *et al.*, 2015; Scheben *et al.*, 2016; Patiño *et al.*, 2017, respectively). This suggests that, currently, no general distribution patterns can be assumed for

Orthotricheae nor for other bryophyte groups, and that bryophytes do not necessarily display larger distribution ranges than angiosperms. Moreover, there is growing evidence that the extended idea on the existence of low levels of endemism in bryophytes needs to be revised.

The results obtained for *Lewinskya acuminata* and *Orthotrichum shevockii* suggest that in both cases the current disjunct distribution might result from long-distance dispersal. In the case of *O. shevockii* this could be tested through dating analyses that placed the disjunction around 0.44–6.67 Ma, dates fitting the origin of the Canary Islands and Tenerife (Fernández-Palacios *et al.*, 2011). This supports the main role of dispersal in the generation of island biodiversity in oceanic archipelagos of volcanic origin (Cowie & Holland, 2006). For *L. acuminata*, no biogeographical analyses could be performed due to the lack of genetic variation between the disjunct populations. However, this fact suggests that a possible origin due to continental drift is quite doubtful, because it would suppose that no genetic variation has occurred since the opening of the Atlantic Ocean, and the split of Europe and North America approximately 40 Ma. ago. Moreover, the limited degree of differentiation found in *L. acuminata* could reflect a recent origin of the disjunct populations, as suggested for other species also present in western North America and the Mediterranean (Shaw *et al.*, 2003). Otherwise, that lack of differentiation could also indicate an ongoing genetic exchange among the different disjunct areas (Shaw *et al.*, 2014). Both hypotheses require further investigations, and different loci or techniques should be employed to check if the molecular homogeneity obtained across distant populations is not only due to methodological shortcomings.

In the case of *Lewinskya affinis*, none of the species within the complex displayed a disjunct distribution. However, this does not discard that the origin of the different species could have been due to long distance dispersal events, as it has been reported for the sister species *Orthotrichum underwoodii* and *O. handiense* (Patiño *et al.*, 2013), also displaying the same disjunction as *O. shevockii*, or in other bryophyte groups (for review see Carter *et al.*, 2017). This could indicate that dispersal may not routinely occur after the speciation process (Crisp *et al.*, 2011), suggesting also that the dispersal capacities of bryophytes could be in some cases lower than expected, or that effective long distance dispersal events might actually be very rare and stochastic (Nathan, 2006; Crisp *et al.*, 2011).

One factor that could affect bryophyte dispersal is the size of the spores. The three species studied here have a spore size range that includes spores smaller than 20 μm,

suitable for long distance dispersal by wind and air currents (Gillespie *et al.*, 2012; Wilkinson *et al.*, 2012). The strategy of spore release in bryophytes has been also related to dispersal. The studied species show the two dispersal mechanisms present in mosses: the xerocastique one (i.e., capsules open when air conditions are dry), in *Orthotrichum shevockii* and *Lewinskya affinis s.str.* and the rest of species of the complex, and hygrocastique (i.e., capsules open when air conditions are wet) in *L. acuminata*. Spore release in wet conditions could imply short distance dispersal, since spores tend to aggregate and to be deposited near the source (Mueller & Neumann, 1988); while in dry conditions spores are more easily dispersed at longer distances by wind or air currents. Interestingly, in the present study, none of the xerocastique species of the *L. affinis* group shows a disjunct distribution. However, both *O. shevockii*, also xerocastique, and *L. acuminata*, hygrocastique, show trans-oceanic ranges. This suggests that long-distance dispersal among the studied species can occur independently of the type of spore release mechanism. Notably, the hygrocastique mechanism of *L. acuminata*, is accompanied by a dual dispersal strategy that could allow the species to disperse across long distances. This moss produces two types of spores: bicellular ones, endosporically germinated and potentially ready to establish just after spore release in wet conditions, but also smaller and normal spores that can be dispersed once desiccated, when the air conditions are dry, being more likely to disperse through long distances. Besides, some other factors might drive the effectiveness of long distance dispersal events. The studies of van Zanten (1978) and van Zanten & Pócs, (1981) correlated different distribution ranges of widespread and endemic bryophytes with differences in the spore survival during transport and establishment (desiccation, UV and freezing resistance). These features should be analyzed and compared for all the considered species in this work together with other xerochastique and hygrochastique taxa of Orthotricheae. Additionally, other ecological factors related to the capacity for colonization and competition that could help to understand the different distributions patterns (Medina N.G. *et al.*, 2011) could be considered. Successful colonization and establishment are more likely when disjunct areas have similar suitable habitats (Gillespie *et al.*, 2012). This fact has been corroborated for *O. shevockii*, and moreover, its restriction to Tenerife Island in Macaronesia, might be due to the lack of similar habitats in the rest of the archipelago. Likewise, *L. acuminata*, colonizes Mediterranean climate areas in both sides of the western Palearctic and western Nearctic disjunction. Besides, its presence in East Africa agrees with the idea that taxa from higher latitudes colonize higher elevation habitats in

regions at lower latitudes (Gillespie et. al., 2012), since the East African populations of this moss were located in mountains above 3,500 m. a.s.l., while in California and the Mediterranean it occurs below 2,000 m. a.s.l.

With respect to the origin of the disjunctions, only that of *Orthotrichum shevockii* could be tested with molecular data. Ancestral area estimations placed the origin of *O. shevockii* in western North America, closely related to other endemic species of that region. These species include *O. underwoodii,* whose sister species *O. handiense* is a Macaronesian microendemism, only present in the Jandia Peninsula of Fuerteventura Island (Patiño *et al.,* 2013). These results support the link between California and Macaronesia, and add evidence to the fact that some bryophyte groups or species from Macaronesia are more connected to America than to the closer continental areas of Africa and Europe (Vanderpoorten *et al.,* 2011). This connection could be promoted by the air currents of the eastward subtropical jet stream that crosses over both California and the Canary Islands (Kuang *et al.,* 2014), as has already been proposed for other bryophyte species showing disjunctions that affect North America and Europe (Frahm 2008). Wind current models testing this distribution pattern, such as those performed by Muñoz *et al.* (2004) in the Southern Hemisphere, could confirm this hypothesis. Furthermore, they could also help to elucidate the exact phylogeographic history of *Lewinskya acuminata*. This species is widespread in the Mediterranean basin and the Canary Islands. However, it is less common in East Africa and western North America, where it has only been found in southern California and northern Ethiopia respectively, and not in neighboring regions also recently surveyed by our research team. This fact could point to the origin of the species in the Mediterranean, but phylogeographic analyses are necessary to test this hypothesis and to evaluate if wind and long distance dispersal are the processes shaping the distribution range of *L. acuminata*. Within the *L. affinis* complex, the biogeographical processes linked to its diversification might be related to vicariance through ancient fragmentation for the western Palearctic and western Nearctic disjunction, or with dispersion across the Atlantic Ocean, including the colonization of the volcanic Canary Islands. The presence of *L. leptocarpa* in East Africa might be related with one long distance dispersal event, or with a stepping stone process, although this latter is less probable for organisms with high dispersal capacities (Gillespie *et al.,* 2012). Moreover, the extremely low molecular differentiation between *L. affinis* and *L. leptocarpa* could suggest a recent origin for the species. Thereby, further studies could elucidate the

direction and timing of the disjunctions involved in the current distribution of the species of the *L. affinis* complex.

Useful molecular markers for species delimitation and biogegraphic studies in bryophytes.

Integrative taxonomy requires combining different sources of information, especially morphological and molecular data (Will *et al.,* 2005; Dayrat, 2005). In bryophytes, molecular tools are still less developed than in vascular plants. Moreover, in some groups of bryophytes, species identification and delimitation based on morphology are still problematic. In this context, describing easy-to-use DNA "barcodes" can be a useful tool for specimen identification when key morphological characters lead to uncertain results. Furthermore, molecular tools are currently the only basis for testing biogeographic hypotheses, since dating analyses, estimation of ancestral areas and diversification rates, or phylogeographic analyses rely on molecular data. Moreover, genetic studies at different levels, including those among populations, are important for species conservation purposes. Thus, finding molecular markers with resolution at intraspecific and population level is an urgent need.

Throughout the studies included in this thesis, we have tested several markers previously identified as appropriate for species delimitation in *Orthotrichum* (Medina R. *et al.,* 2012, 2013) and other groups of bryophytes (Stech & Quandt, 2010; Renner *et al.,* 2013), as well as for phylogeographic analyses (Mc. Daniel and Shaw, 2003; Laenen *et al.,* 2011; Pisa *et al.,* 2013, 2014). However, they have shown very low levels of variation within *Orthotrichum* and *Lewinskya* and very low resolution at intraspecific level. This latter case was especially remarkable in *L. acuminata*, where disjunct populations were resolved in a politomy in phylogenetic analyses with the combined loci dataset, impeding the performance of dating analyses to test the origin of the disjunction. Our study confirms that the most commonly used markers within bryophyte phylogenetic studies are not always useful when working with closely related species or with species complexes as suggested by other authors (Hassel *et al.,* 2013; Stech *et al.,* 2013). This shortcoming requires our attention when intending to use them as DNA barcodes, since one premise for that is universality. Moreover, markers like ITS2, *rps*4, *atp*B *or trn*L-F, that have proved to be useful for phylogeographic studies in different bryophyte groups, have provided less informative results when applied to *Orthotrichum* and *Lewinskya*. This

reveals that each genus or group of species has its own features with respect to molecular variation. The markers used in *L. affinis*, especially the nuclear *ETS-115* and *ETS-317* regions from Mc. Daniel *et al.* (2013), resulted to be useful for species delimitation. However, the rate of amplification for these *ETS* regions was considerably low, even when using fresh materials in good conditions (83 and 71 obtained sequences from the total 115 included), so they might be discarded as possible DNA barcode regions, at least for Orthotricheae, since other premises for barcoding are high amplification and sequencing success (Hollingsworth *et al.*, 2009).

Thus, the results of chapters 3.1, 3.3 and 3.4, emphasize the need of using different approaches like genome wide molecular and Next-Generation Sequencing approaches in order to search for new and specific variable regions (Liu *et al.*, 2012), or tools that allow the use of the complete organellar genomes to perform studies at intraspecific level (Lewis *et al.*, 2016). This seems to be especially necessary for phylogeographic objectives within Orthotricheae. However, in chapter 3.2, the comparison of two mitochondrial genome sequences of *Orthotrichum diaphanum* from different populations failed to reveal suitable variable regions at intraspecific level, since the variation was low and dispersed along the genome, which impeded the design of primers. On the contrary, when comparing the sequences of *O. diaphanum* with that of *O. macrocephalum* and other species of Orthotricheae, the complete mithochondrial genomes were useful for phylogenetic purposes. The results supported the polyphyly recovered for *Orthotrichum s.l.*, sustaining its recent proposed subdivision into two genera: *Orthotrichum* and *Lewinskya* (Goffinet *et al.*, 2004; Lara *et al.*, 2016).

Ongoing projects and future perspectives

From the analysis of the results obtained in this study, it is clear that the real diversity of the Orthotricheae, and by extension of the whole family, is still far from being fully known. Up to now the studies in this tribe using integrative taxonomy approaches have focused on the Northern Hemisphere, and although Holarctic disjunctions could be still evaluated for other species, it would also be interesting to assess if similar results would be obtained for the Southern Hemisphere with respect to species diversity and their actual ranges of distribution. This work evidences the need to continue studying in detail those species that show wide distributions, and whose morphological variation is considered

wide along their distribution ranges, and in particular in cases of intercontinental disjunctions.

In the case of the species included in the present work, phylogeographic studies are still pending for *Lewinskya acuminata* and *Orthotrichum shevockii*. In the first case, in order to establish the origin and age of that disjunction between the Mediterranean, California and East Africa, and to test the hypothesis of long-distance dispersal as the process most likely explaining its distribution. Population genetic studies in both cases could allow to assess if gene flow is maintained along these distributions, and in the case of *O. shevockii*, to evaluate the status of the Canarian populations and to consider conservation strategies if necessary, given their restricted distribution to the highlands of the island of Tenerife. Related to the *L. affinis* complex, further analyses are needed to discern the biogrographical history of this group, and to assess if the low molecular differentiation found between *L. affinis* and *L. leptocarpa*, is due to a recent origin of the latter or to methodological shortcomings regarding the molecular regions used.

In order to perform these analyses, it is first necessary to find molecular markers variable and informative enough at intraspecific level. Given the scarce variation observed between species for the markers used in this study, the difficulties to amplify and sequence some of the new markers tested, as well as the low variation obtained for the mitochondrial genome, new alternatives should be tested. One option would be to continue exploring different NGS tools, focusing on the plastid and nuclear ribosomal DNA genomes, and testing methodologies such as restriction-site associated DNA sequencing (RAD-Seq) and genotyping-by sequencing (GBS), whose develop and use is increasing.

Conclusions

1. *Lewinskya acuminata* is addressed as a widespread taxon, expanding outside the Mediterranean Basin and Macaronesia, with disjunct populations in western North America (California) and East Africa (Ethiopia).

2. The intercontinental distribution of *Lewinskya acuminata* contrasts with its hygrochastique character. It might be favoured by having a dual strategy for spore production. Spore release in wet conditions might allow the bicellular spores to quickly germinate and establish, while the small spores additionally produced might be dispersed by air when the conditions are drier, therefore leading to reach longer distances.

3. *Orthotrichum shevockii* is a widespread species in California. The new populations of this moss found in the Canary Islands (Tenerife) confirm a trans-oceanic distribution, showing a disjunction that most probably took place in the early Miocene–Pliocene (2.74 Ma) from North America to the Canary Islands.

4. The up-to-date concept of *Lewinskya affinis* actually comprises seven different species that include two reinstated taxa, *L. fastigiata* and *L. leptocarpa,* and four new species, *L. arida, L. pacifica, L. pseudoaffinis* and *L. scissa.* All of them are sibling species, and together with *L. praemorsa* and *L. tortidontia* constitute a monophyletic natural group with very similar morphologies.

5. The intercontinental distribution of *Lewinskya affinis* is discarded, for being a species restricted to Europe and the Mediterranean basin. None of the remaining species in the *L. affinis* complex has a disjunct distribution. The range of *L. fastigiata* largely overlaps that of *L. affinis*; *L. leptocarpa* is restricted to East Africa; *L. scissa* is only reported from Gran Canaria (Canary Islands), and the other three species -*L. arida*, *L. pacifica,* and *L. pseudoaffinis*- are sympatric and restricted to western North America.

6. All the studied species match the western Palearctic – western Nearctic disjunction. In the case of *Orthotrichum shevockii,* the eastern side of the disjunction only concerns Macaronesia, a pattern that is very rare among bryophytes. The distribution ranges of *Lewinskya acuminata* as a single species, and the *Lewinskya. affinis* complex as a whole, exceed the mentioned disjunction since in both cases they extend to East Africa

(Paleotropical region), which constitutes a third continental disjunct area and implies an infrequent biogeographic pattern.

7. Bryophyte species display different distribution patterns even within a same tribe, as is the case of Orthotricheae, where contrasting situations are not rare. In fact, situations including species that show intercontinental disjunctions together with others having restricted distributions like those reported in this work and other previous ones prevent against establishing generalized patterns of distributions within this tribe, but also among bryophytes.

8. Long distance dispersal plays an important role in shaping the distributions of the species of Orthotricheae, as has been suggested for some other groups of bryophytes. It relates with the origin of the disjunction of *Orthotrichum shevockii*, and most probably also at that of *Lewinskya acuminata*. Similarly, it might underlie the current distribution of the *Lewinskya affinis* complex. However, further analyses are needed to clarify the biogeographical history of *L. acuminata* and the diversification process within the *L. affinis* complex.

9. *Orthotrichum* and *Lewinskya* usually show little variation in the molecular regions most commonly used in bryophytes studies, especially at intraspecific level. The new markers used in this study have been useful for species delimitation, but they have been unsuccessful at population level, and the amplification rates of the nuclear ones have not been satisfactory enough.

10. The comparison of the mitochondrial genome of *Orthotrichum diaphanum* and *O. macrocephalum* has revealed some regions identified as potential new markers for phylogenetic and species delimitation studies. However, the intraspecific molecular variation of *O. diaphanum* is relatively low and dispersed, which hampers the design of specific markers for phylogeographic and population analyses within the Orthotricheae. Further research is needed to detect more reliable markers at intraspecific level.

11. The phylogenies obtained combining different nuclear and chloroplast loci and the one performed with the complete mitochondrial genome support the recently proposed division of *Orthotrichum* s.l. into the genera *Orthotrichum s.str.* and *Lewinskya*, as well as the closest relation of the latter with *Ulota*.

12. The taxonomic results of this thesis include the discovery of seven species within *Lewinskya affinis* s.l., four of them new to Science, and the synonymyzation of *Orthotrichum kellmanii* with *Orthotrichum shevockii,* which evidence that the diversity and taxonomy of the tribe Orthotricheae are still far from being completely known.

13. Cryptic speciation and taxonomic shortcomings are among the factors that point to an underestimation of the rates of endemism in bryophytes. The existence of widely and narrowly distributed species in the same genera as confirmed in this work, makes necessary additional studies to assess the true importance of the different patterns of distribution in bryophytes.

14. Integrative taxonomy has proved to be, once more, an optimal methodological framework to address biodiversity and biogeographic studies in bryophytes. In the three studied cases, results from morphological and molecular analyses have been congruent and mutually essential in order to establish the limits of the studied species, as a necessary step to analyze their phylogenetic relationships and distributions patterns.

Conclusiones

1. *Lewinskya acuminata* es un taxón ampliamente distribuido, que se expande fuera de la cuenca mediterránea y Macaronesia, con poblaciones disyuntas en el oeste de América del Norte (California) y África oriental (Etiopía).

2. La distribución intercontinental de *Lewinskya acuminata* contrasta con su carácter higrocástico. Esto podría estar favorecido por el hecho de presentar una peculiar estrategia en la producción de esporas, consistente en liberar esporas unicelulares pequeñas y otras bicelulares de mayor tamaño. La liberación de esporas en condiciones húmedas podría permitir por un lado que las esporas bicelulares germinen y se establezcan rápidamente, mientras que por otro las esporas de menor tamaño podrían dispersarse por aire cuando las condiciones son más secas, pudiendo alcanzar distancias más largas.

3. *Orthotrichum shevockii* es una especie muy extendida en California. Las nuevas poblaciones de este musgo que se han encontrado en las islas Canarias (Tenerife) confirman que tiene una distribución transoceánica, disyunción que probablemente tuvo lugar en el Mioceno-Plioceno temprano (2.74 Ma) desde Norteamérica hacia las Islas Canarias.

4. El concepto actual de *Lewinskya affinis* es en realidad un complejo de siete especies diferentes que incluye dos taxones recuperados a nivel de especie, *L. fastigiata* y *L. leptocarpa*, y cuatro especies nuevas, *L. arida, L. pacifica, L. pseudoaffinis* y *L. scissa* Todas ellas son especies hermanas que, junto con *L. praemorsa* y *L. tortidontia,* constituyen un grupo natural monofilético de especies con morfologías muy similares.

5. Se descarta la distribución intercontinental de *Lewinskya affinis*, por ser una especie restringida a Europa y la Cuenca mediterránea. Ninguna de las demás especies del complejo de *L. affinis* presenta una distribución disyunta. El rango de distribución de *L. fastigiata* se superpone con el de *L. affinis*; *L. leptocarpa* está restringida a África Oriental; *L. scissa* sólo se ha encontrado en Gran Canaria (Islas Canarias), y las otras tres especies -*L. arida, L. pacifica* y *L. pseudoaffinis*- son especies simpátricas y su distribución está restringida al oeste de Norteamérica.

6. Todas las especies estudiadas están relacionadas con la disyunción Paleártico Occidental - Neártico Occidental. En el caso de *Orthotrichum shevockii*, el extremo oriental de la disyunción sólo afecta a Macaronesia, un patrón de distribución muy raro entre los briófitos. El rango de distribución de *Lewinskya acuminata*, y del complejo de *Lewinskya affinis* en su conjunto, excede la disyunción mencionada ya que en ambos casos el rango de distribución se extiende a África Oriental (región Paleotropical), lo que constituye una tercera zona continental disyunta, determinando un patrón biogeográfico infrecuente entre los briófitos.

7. Las especies de briófitos muestran diferentes patrones de distribución incluso dentro de una misma tribu, como es el caso de Orthotricheae, donde no es raro encontrar situaciones opuestas. De hecho, en este trabajo y en otros anteriores, se han descrito situaciones que incluyen especies con disyunciones intercontinentales junto con otras que tienen distribuciones restringidas. Ello impide por tanto establecer patrones generalizados de distribución dentro de esta tribu y de los briófitos en general, hasta que se disponga de suficiente información sobre la distribución de todos o de la mayoría de sus integrantes.

8. La dispersión a larga distancia juega un papel importante en la configuración de las áreas de distribución de las especies de Orthotricheae, como se ha sugerido a su vez para otros grupos de briófitos. Este mecanismo está en el origen de la disyunción de *Orthotrichum shevockii*, y muy probablemente también con el de *Lewinskya acuminata*. Asimismo, la dispersión a larga distancia podría tener relación con el origen de la distribución actual de las especies del complejo de *Lewinskya affinis*. Sin embargo, son necesarios más estudios que permitan aclarar la historia biogeográfica de *L. acuminata*, así como el proceso de diversificación dentro del complejo de *L. affinis*.

9. *Orthotrichum* y *Lewinskya* muestran generalmente poca variación en las regiones moleculares más comúnmente usadas en estudios de briófitos, especialmente a nivel intraespecífico. Los nuevos marcadores utilizados en este estudio han sido útiles para la delimitación de especies, pero no han tenido éxito en el ámbito poblacional y las tasas de amplificación de las regiones nucleares no han sido suficientemente satisfactorias.

10. La comparación del genoma mitocondrial de *Orthotrichum diaphanum* y O. *macrocephalum* ha permitido identificar algunas regiones de este genoma como potenciales nuevos marcadores para realizar estudios filogenéticos y de delimitación de especies. Sin embargo, la variación molecular intraespecífica en *O. diaphanum* es

relativamente baja y dispersa, lo que dificulta el diseño de marcadores específicos para estudios filogeográficos y de análisis de poblaciones dentro de Orthotricheae. Esto hace necesaria la realización de nuevos estudios que permitan descubrir marcadores más informativos a nivel intraespecífico.

11. Las filogenias obtenidas combinando diferentes regiones del núcleo y del cloroplasto, así como la realizada con el genoma mitocondrial completo, apoyan la reciente propuesta de división de *Orthotrichum s.l.* en los géneros *Orthotrichum s.str.* y *Lewinskya*, así como la relación más próxima de este último con *Ulota*.

12. Los resultados taxonómicos de esta tesis incluyen el descubrimiento de siete especies dentro de *Lewinskya affinis* s.l, cuatro de ellas nuevas para la ciencia, y la sinonimización de *Orthotrichum kellmanii* con *Orthotrichum shevockii*, lo que evidencia que la diversidad y taxonomía de la tribu Orthotricheae están aun lejos de ser completamente conocidas.

13. La existencia de especies crípticas y los errores o malinterpretaciones taxonómicas son algunos de los factores que apuntan a una subestimación de las tasas de endemismo en briófitos. La presencia de especies con rangos de distribución amplios o restringidos dentro de un mismo género, como se ha confirmado en este trabajo, hace necesaria la realización de nuevos estudios que permitan evaluar la verdadera importancia de los diferentes patrones de distribución dentro de los briófitos.

14. La taxonomía integrativa ha demostrado ser, una vez más, un marco metodológico adecuado y necesario en el estudio de los briófitos para abordar cuestiones relacionadas con la biodiversidad y la biogeográfia. En los tres casos estudiados, los resultados de los análisis morfológicos y moleculares han sido congruentes y mutuamente esenciales para establecer los límites de las especies implicadas, como un paso necesario para analizar sus relaciones filogenéticas y patrones de distribución.

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Agradecimientos

Llegar hasta aquí ha sido un camino largo, a veces tortuoso y cuesta arriba, nadie dijo que fuera a ser fácil esta aventura, pero gracias al apoyo de la familia, amigos, compañeros y muchos otros que se han ido uniendo en cada etapa, habéis hecho posible que esos tramos fueran mucho más fáciles, además de disfrutar juntos de los buenos momentos del camino y de la llegada a la cima. Y ahora toca celebrarlo y agradecéroslo.

En primer lugar, agradecer a mis directores de tesis, Vicente, Paco e Isabel, por darme la oportunidad de embarcarme en este proyecto, y de adentrarme en este complejo e intricando mundo de los briófitos. Gracias por confiar en mí y por todo lo aprendido en estos años. Y gracias por darme la oportunidad de conocer mundo en los viajes de muestreo que hemos realizado, el Sur de California y Baja California, Etiopía, Kenia y Tanzania, que han dado, y darán, muy buenos frutos científicamente, pero también buenos momentos disfrutando de unos lugares y gente tan increíbles.

Gracias a Alain y a Bernard por acogerme durante las dos estancias de la tesis en Lieja y Connecticut, por integrarme en el equipo como si fuera una más, y por todo lo aprendido durante ese tiempo. Gracias a Jairo por el tiempo dedicado a sacar adelante la parte molecular de *L. affinis* que tanto se atascó, y por enseñarme nuevos análisis que le han dado otra perspectiva a esta tesis. A Yang, por su paciencia en el laboratorio con los eternos protocolos para secuenciar genomas y por enseñarme a lidiar con el análisis de los datos. Y también gracias a Rafa y a Marta por su ayuda para alinear los genomas y no morir en el intento.

Gracias también a mis compañeras Nagore y Rut, y a Rafa, por todos los consejos, y por estar ahí en esos momentos iniciales en los que uno anda tan perdido, y por su puesto a lo largo de esta tesis. Gracias Nagore por tu ayuda con "R", por ayudarme a abrirme camino en este mundo de códigos y scripts que aún me trae de cabeza, con tus enseñanzas he conseguido sacar adelante los análisis estadísticos y además rematarlos con unos gráficos la mar de resultones con los programas que me has descubierto. Agradecer también a Virginia, por todas las dudas que me has resuelto tanto en el laboratorio como en los análisis moleculares y estadísticos, y que me han sacado de más de un atolladero, y por descubrirme Geneious. A Richi, por todas las correcciones y comentarios tan útiles sobre los artículos, por las fotos al microscopio electrónico y por acogerme aquellos días en Valencia y mirar mano a mano nuestros queridos "*affine*". A Ángela por echarme una mano introduciendo los pliegos de la tesis en el herbario. Y a todos los compañeros de la Unidad de Botánica de la UAM con los que he podido compartir las clases de prácticas y salidas de campo. Y en especial a la "mesa camilla", aunque ahora ya muy mermada, estos años hemos compartido grandes tertulias a la hora de comer, y por supuesto nuestra pasión por lo dulce. Fátima, Nagore, Rut, Belén, Isa, Rafa, Manolo, Helios, Fernando, María y los que han pasado periódicamente por allí en estos años. Gracias.

Y no me puedo olvidar de todos los guías y el personal que nos han acompañado en los viajes de muestro en África. Biniam fue de gran ayuda en Etiopía, además de llevarnos de un lado a otro, fue sin pedirlo, un guía de fauna magnífico y un gran compañero de viaje, y desarrolló toda una técnica para secar los sobres de muestras dentro del coche. En el monte Kenia y el Kilimanjaro tuvimos dos equipos increíbles, gracias a ellos por facilitarnos la ruta y por el duro trabajo que realizan. De este último viaje aprendí que siguiendo el mantra del "pole pole sana" cualquier meta o cima se alcanza.

Dicen que la familia no se elige, pero los amigos sí. Pues yo sin duda hice en su día hace más de veinte años la mejor elección. Lys, gracias por tus correcciones en esta fase final de la tesis, por dedicarle tiempo a leer sobre mis musgos, por resolver mis dudas con el inglés mientras escribía, por seguir algunas de mis locuras. Pero sobre todo estar siempre ahí, ya sea a kilómetros de distancia o separadas por un tabique, compartiendo penas y alegrías. Gracias. Cristian, aún recuerdo cuando al acabar la carrera decías que no nos íbamos a volver a ver. Y aquí seguimos aguantándonos unos cuantos años después, y compartiendo viajes y planes, pajareo y petardeo, y horas bajo el sol buscando tus Ricotís, así que aún me debes unos cuantos musgos a cambio. Pablo, aún tenemos pendiente volver a hacer un viajecito de los nuestros, ya sea en Oxford o alrededores, o donde se tercie, y por supuesto algún otro concierto de Tarque *et al*., que no hay que perder las buenas costumbres. Gracias por seguir ahí después de tantos años. Antonia, digo Fátima, gracias por ser como eres, por sacar la locura creativa que tenemos dentro juntas, por compartir tanto, por endulzarme la vida con golosadas varias, por ser mi apoyo estos años en la UAM pero también fuera de ella, por dejarme "arrastrar al lado oscuro" a tus increíbles retoños. Te voy a echar mucho de menos. Pero nos juntaremos, aunque sea para montar fiestones de los nuestros, y cuando Alaia y Altair empiecen a pasar de nosotras, ya buscaremos otras excusas.

Por raro que parezca, tengo que darle las gracias a *WhatsApp*. Esta tesis no hubiera sido lo mismo estos tres últimos años sin su existencia, porque el grupo de "Gente Bizarra Vicariante" ha sido uno de los grandes apoyos desde la distancia. Ya en sus inicios en la cafetería de Biología de la UAM servía de escape y desconexión, nuestro gran foro de discusiones, de las buenas, de los temas más variopintos. Pero a medida que el éxodo que sufren los biólogos en este país hacía mella en el grupo, *WhatsApp* nos ha seguido juntado y nuestras tertulias no han disminuido de intensidad. Ya sea para resolver el dilema atún/bonito, llorar penas y alegrías, solucionar dudas, papeleos, o compartir los chistes más absurdos. Gracias Pablo, Lys, Mike, Isidro, Espe, Raúl y Nacho, gracias Gentes Bizarras Vicariantes por estar ahí, virtual o personalmente cuando logramos juntarnos. Ana, aventurera incansable, gracias por tu amistad, tus consejos y tu sabiduría, por tus risas y por tus visitas improvisadas, siempre bien recibidas, sabes que allá donde esté tienes un hueco para ti. Javi, a pesar de que hayas accedido a resolver mis dudas sobre estadística sólo por el hecho de que recibías a cambio chocolate, gracias por dejarte sobornar. Gracias por soportarme estos años, aunque para tanto

no habrá sido que ahora hasta somos pareja de baile. Quién nos lo iba a decir cuando nos conocimos en Monticola. Y hablando de Monticola, daros las gracias a todos los monticoleños de los que he aprendido tanto, especialmente a las amistades que han surgido con los años de compartir pajareo y alguna que otra jornada de anillamiento: Javi, Espe, Carlos, Álvaro, Jorge, Moni, Pablo, Alberto, Adri, Cristian, Juancho. Y gracias a mis polluelos por aguantarme estos dos últimos años, sois muy grandes. Y a los compañeros de carrera, Aroa, Argentina, Ángeles, Pablo, Cristian, Raul, Eva, Victor, Miguel, Rosa, Javi, ahora tan desperdigados por la geografía española o fuera de sus fronteras, con los que he aprendido a ser bióloga o al menos intentarlo, entre clases, prácticas, sala de estudio, juergas, viajes. Gracias por los momentos compartidos.

Jorge, Monica, Érika y Lys, mejores compañeros de piso no podría haber tenido, algunos ya erais amigos de los de toda la vida, a otras nos juntó el compartir techo y peleas con los caseros, pero os habéis convertido en grandes amigas y apoyo en estos cuatro años. Rafa, Alfredo y Marta, gracias por ser mi familia durante mi paso por Connecticut, por todas las cenas, excursiones y charlas compartidas, que de tanto me han servido para afrontar la etapa final de la tesis de otra manera. Espero que los *Uconn Spaniards* podamos juntarnos pronto. Y de mi etapa en Connecticut me llevo también un gran recuerdo de los demás compañeros del "Goffinet Lab", Dinah, Antoine, Satpal, Yang, Lily y Nasim, especialmente del viaje de acampada al Monte Whasington; y de Eric y Stella que me abrieron las puertas de su hogar y me trataron como si fuera de la familia. Un placer compartir vuestra casa y vuestra compañía. *Thank you.* Como en casa me sentí también en Lieja, *merci* Elza, mi "abuela" Belga, con sus exquisitos platos y postres caseros, y por animarme a retomar el francés y por esas conversaciones tan interesantes con Adelka y Edmond. Y allí en Lieja me inicié en el swing, y conocí a gente estupenda como Esther, Katia o Benjamin. Y de Bélgica a España, el swing ha seguido en mi vida, una gran vía de escape, y de mi paso por Big South me llevo especialmente a Diego, Edu y Sara, gracias por todos esos bailes, y los que nos quedan, y por nuestras conversaciones mojito en mano.

Gracias a mi otra familia, porque en estos años me habéis dado muy buenos momentos. Araceli, Juan, Gus, Eva, y los seis terremotos de sobrinos, Jesús, Nahia, Hugo, Mario, Leo y Mariana. Gracias. Y gracias Gus por tus consejos a última hora peleándome con la maquetación de la tesis.

Juancho, esta tesis se debe en gran parte a ti. Hace algo más de nueve años coincidimos en una excursión de Monticola, y me desviaste sin tu saberlo del camino de la biología celular, al redescubrirme la parte de la biología de bota que en el fondo tanto me gustaba. Gracias por enseñarme tantas cosas todos los días. Gracias por confiar en mí y por estar a mi lado, por compartir tanto y por seguir haciéndome reír cada día. Durante estos años has sido mi apoyo incondicional, y a pesar de la distancia, esta tesis la has vivido y "sufrido" casi casi igual que yo, y ha salido de algunos cuantos

baches gracias a ti. Además, nos ha permitido descubrir mundo juntos, y subir a la cima más alta que he alcanzado, literalmente en el Kilimanjaro, pero también en lo personal. Te quiero.

Y por último darle las gracias a mi familia, porque sin ellos no habría llegado hasta aquí. Por todo el apoyo que me han dado, y siguen dando día a día. A mis padres, por la importancia que le han dado siempre a nuestra formación, y por enseñarme que hay que aprender de todo, porque nunca sabes qué puertas se van a abrir. Pero lo que más destaca de mi familia, de todos y cada uno de ellos, es el enseñarme a luchar por lo que uno quiere, por lo que uno cree y a defenderlo, y que juntos no nos moverán. Y aunque os lo diga poco, os quiero.

A todos, gracias por haber hecho de este camino una gran aventura.